DEPARTAMENTO DE CIÊNCIAS DA VIDA
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Brain Dysfunction and Cell Death in Type 2 Diabetes: A neuroprotective role for the peripheral treatment with Exendin-4

Inês Carolina de Sousa Madeira e
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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Doutora Ana Isabel Duarte (Universidade de Coimbra)

Inês Carolina de Sousa Madeira e Antunes Sebastião

2014

It always seems impossible until it’s done.

Nelson Mandela

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Resumo

A Diabetes tipo 2 e a doença de Alzheimer são, nos dias de hoje, dois graves problemas de saúde pública. A diabetes tipo 2 afecta milhões de pessoas em todo o mundo e os seus números continuam a aumentar. Esta epidemia tem grandes repercussões no quotidiano dos pacientes, maioritariamente devido às suas complicações crónicas que podem afectar não apenas vários órgãos periféricos, mas também o cérebro, constituindo inclusivamente um factor de risco para o desenvolvimento da doença de Alzheimer. Esta é a forma de demência mais comum, na qual a resistência à insulina (uma das características da diabetes tipo 2) tem sido amplamente descrita, pelo que tem sido colocada a hipótese de que a doença de Alzheimer e a Diabetes tipo 2 serão mais do que meras patologias não interrelacionadas. De facto, um número crescente de evidências científicas sugere a existência de inúmeros elos moleculares de ligação entre as duas patologias (*ex*.: dismetabolismo da glucose, insulina e disfunção mitocondrial, stresse oxidativo), que poderão culminar em morte celular e consequente disfunção cerebral. Assim, a doença de Alzheimer tem sido cada vez mais referida como uma "diabetes tipo 3" ou uma "diabetes tipo 2 específica do cérebro". Nesta perspectiva, tem sido recentemente sugerido que fármacos eficientes contra a diabetes tipo 2 poderão também constituir uma estratégia terapêutica contra a demência (particularmente contra a doença de Alzheimer). Um dos fármacos anti-diabéticos potencialmente promissores é a exendina-4, pertencente à classe das incretinas/agonistas dos receptores do GLP-1 (correspondente a *glucagon-like peptide*-1) e já utilizado clinicamente no tratamento da diabetes tipo 2. Além do seu efeito insulinotrópico a nível periférico, por se tratar de um fármaco sintético do tipo das incretinas, a exendina-4 possui a capacidade de induzir o chamado "efeito incretina" (promove a secreção de insulina dependente de glucose), sendo resistente à degradação por parte da enzima dipeptidil peptidase-IV e diminuíndo o risco de hipoglicemia frequentemente associado aos fármacos anti-diabéticos "tradicionais". Adicionalmente, tem sido descrito que a exendina-4 reduz a ingestão de alimentos e, desta forma, poderá constituir uma terapia anoréctica eficaz. Mais relevante para este estudo parece ser o número crescente de evidências de que a exendina-4 poderá desempenhar também um papel neuroprotector, estando inclusivamente a decorrer vários ensaios clínicos neste sentido. Contudo, os mecanismos moleculares precisos subjacentes à neuroprotecção pela exendina-4 contra a disfunção cognitiva associada a diabetes tipo 2 e doença de Alzheimer continuam por esclarecer.

Desta forma, no presente estudo colocámos a hipótese de que a exendina-4 melhora as alterações periféricas associadas à diabetes tipo 2, bem como dos mecanismos intracelulares de controlo de qualidade no cérebro, neurodegeneração e morte celular, melhorando assim a função sináptica e, em última análise, a função cognitiva em condições de diabetes tipo 2. Assim, tivemos como objectivo principal analisar o potencial efeito neuroprotector da administração contínua, crónica e periférica com exendina-4, utilizando para este efeito o cérebro de ratos meia-idade, diabéticos tipo 2. Mais especificamente, avaliámos o papel da administração crónica periférica de exendina-4 em ratos diabéticos tipo 2, ao nível dos seguintes parâmetros periféricos: *1)* características patológicas (níveis sanguíneos de glucose, taxa de remoção de glucose do sangue, níveis de insulina e hemoglobina glicosilada no plasma e resistência à insulina); *2)* outros factores de risco associados à diabetes tipo 2 (como o peso corporal, hipercolesterolémia e elevada pressão sanguínea), assim como em amostras de córtex cerebral: *3)* marcadores de stresse oxidativo, *4)* (dis)função sináptica, *5)* mecanismos de controlo de qualidade celular (autofagia) e *6)* neurodegeneração e morte celular necrótica e apoptótica (dependente e independente de caspases).

No presente estudo foram utilizados homogeneizados de córtex cerebral de ratos não-diabéticos Wistar e

diabéticos tipo 2 não-obesos Goto-Kakizaki, ambas as estirpes correspondiam a ratos de 8 meses, submetidos continuamente (ou não) à administração periférica de exendina-4 (5 μg/kg/dia, velocidade de infusão de 2.5 μl/h), durante 28 dias, mediante implantação subcutânea de bombas micro-osmóticas Alzet. Os animais foram monitorizados antes e periodicamente durante o tratamento, no sentido de avaliar o seu bem-estar dos animais e o sucesso do tratamento. No final deste, os ratos foram eutanasiados e os parâmetros sanguíneos relacionados com a diabetes tipo 2 e os seus factores de risco mais comuns foram determinados. Adicionalmente, foram preparados homogeneizados citosólicos corticais e respectivas fracções mitocondriais isoladas, de modo a serem utilizados na quantificação dos níveis de GLP-1, insulina e glucose, bem como nos níveis de expressão proteica do receptor do GLP-1 e na activação do receptor de insulina, mediante aplicação das técnicas de ELISA, "western blotting" ou co-imunoprecipitação. Seguidamente, procedeu-se à determinação, por colorimetria, ELISA ou "immunoblotting", de vários marcadores de oxidação de lípidos e ADN, autofágicos, de apoptose dependente e independente de caspases e de necrose. Finalmente, o papel de exendina-4 na função sináptica do córtex de cérebros de ratos diabéticos tipo 2 foi também avaliado por "western blotting".

No presente estudo, observámos que a administração contínua periférica de exendina-4 pareceu suplantar as características patológicas periféricas associadas à diabetes tipo 2 (hiperglicemia, menor taxa de remoção de glucose do sangue, níveis elevados de hemoglobina glicosilada e resistência à insulina). Além disso, a exendina-4 recuperou os níveis de GLP-1 no córtex cerebral de ratos GK e, apesar de não ter mostrado efeitos significativos na expressão proteica do receptor do GLP-1, não descartamos a hipótese de que o fármaco melhore a actividade deste receptor no cérebro. Todavia, a clarificação deste assunto requer estudos adicionais. Surpreendentemente, a exendina-4 não afectou os níveis corticais de insulina nem a activação do seu receptor, sugerindo que este poderia não estar directamente envolvido nos efeitos subsequentes da exendina-4 no cérebro de ratos GK. Tais efeitos incluíram o restabelecimento dos níveis de glucose no cérebro e uma tendência para a exendina-4 proteger contra a oxidação lipídica e do ADN. Notavelmente, os nossos resultados também apontaram para uma activação tendencialmente maior do processo autofágico no córtex cerebral dos ratos GK tratados com exendina-4, provavelmente constituindo uma estratégia protectora contra a morte celular mediada pela caspase-3 associada à diabetes tipo 2 nos ratos GK tratados com solução placebo. De acordo com esta sugestão, a exendina-4 pareceu inibir a apoptose induzida pela caspase 3 no córtex cerebral dos ratos GK diabéticos tipo 2, provavelmente devido à (pelo menos parcialmente) sua tendência para reter a proteína anti-apoptótica Bcl2 e a pro-apoptótico citocromo c na mitocôndria, enquanto a Bax (outra proteína pro-apoptótica) permaneceu no citosol, originando posteriormente uma ligeira melhoria na função cortical dos ratos diabéticos tipo 2 expostos a exendina-4.

Em suma, os nossos resultados sugerem que, através de uma melhoria das características patológicas periféricas da diabetes tipo 2, a terapia crónica com exendina-4 por via subcutânea poderá ter um impacto benéfico no córtex cerebral de ratos machos, não obesos e diabéticos tipo 2 de meia-idade, através do restabelecimento dos níveis de GLP-1 e protegendo contra eventuais danos associados a hiperglicemia local e stresse oxidativo. Essa protecção pela exendina-4 provavelmente envolverá uma estimulação "fisiológica" dos mecanismos de autofagia, com o consequente impedimento da morte celular apoptótica e da disfunção sináptica. Nesta perspectiva, a administração crónica periférica de exendina-4 poderá constituir uma abordagem terapêutica promissora contra as complicações a longo-termo da diabetes tipo 2, nomeadamente as que afectam directamente o cérebro.

Palavras-chave: Diabetes tipo 2, doença de Alzheimer, fármacos anti-diabéticos, incretinas, GLP-1, exendina-4

Abstract

Type 2 diabetes (T2D) and Alzheimer disease (AD) are two major health care issues nowadays. T2D affects millions of people worldwide, whose number continues to increase. This epidemic has major repercussions in the patients' daily life, mainly due to its chronic complications that can affect not only several peripheral organs, but also the brain, and constitutes a risk factor for development of AD. AD is the most common form of dementia, in which insulin resistance (one of main hallmarks of T2D) has been widely described, thus rendering both AD and T2D more than mere unrelated pathologies. Indeed, increasing evidences suggest that T2D and AD share several common molecular links (*e.g.* glucose dysmetabolism, insulin and mitochondrial dysfunction, oxidative stress) that culminate in brain dysfunction and cell death. As such, AD has been increasingly referred to as "type 3 diabetes" or a "brain-specific T2D". In line with this, it has been recently hypothesized that efficient drugs against T2D could be also a beneficial therapeutic strategy against dementia (particularly AD). And one of such potentially promising anti-T2D drugs is exendin-4 (Ex-4), a already clinically used compound from the incretin/glucagon-like peptide-1 (GLP-1) receptor (GLP-1R) agonists class of anti-T2D drugs. Besides its peripheral insulinotropic effect, as a synthetic incretin-like compound, Ex-4 is known to have an incretin effect (promoting a glucose-dependent insulin secretion), being resistant to dipeptidylpeptidase-IV (DPP-IV) degradation and lowering the risk of hypoglycemia, often associated to the "traditional" anti-T2D therapies. Importantly, Ex-4 has been also described as a potential food intake reducer, an anorectic drug and, most importantly herein, a highly promising neuroprotective agent, with clinical trials already ongoing in this line. However, the knowledge on the precise molecular mechanisms underlying such neuroprotection against chronic T2D-associated cognititve dysfunction and AD remain unclear.

Under this perspective, we hypothesized that Ex-4 restores the T2D-associated alterations in intracellular quality control mechanisms, neurodegeneration and death, thereby improving synaptic function and, ultimately, cognitive function. Therefore, our main goal was to study the potential neuroprotective effect of chronic, continuous peripheral Ex-4 treatment in middle-aged T2D rat brain. More specifically, we evaluated the role of chronic peripheral Ex-4 administration in T2D: *1)* pathological features (blood glucose levels, rate of glucose clearance from blood, plasma insulin and glycosylated hemoglobin A_{1C} (HbA_{1C}) levels and insulin resistance); *2)* other T2D-associated risk factors (as body weight, hypercholesterolemia, hypertriglyceridemia and high blood pressure), as well as in *3)* rat brain cortical markers of oxidative stress, *4)* synaptic (dys)function, *5)* cellular quality control (autophagic) mechanisms and *6)* neurodegeneration, caspase-dependent and –independent apoptotic and necrotic cellular death.

Herewith, we used middle-aged (8 month old) non-diabetic male Wistar and non-obese, T2D Goto-Kakizaki (GK) rat brain cortical homogenates, either submitted or not to the continuous peripheral delivery of Ex-4 (5 μg/kg/day, at an infusion rate of 2.5 μl/h), for 28 days, upon subcutaneous implantation of micro-osmotic Alzet pumps. Animals were monitored before and periodically during treatment to determine animals' welfare and treatment's success. At the end of treatment, rats were euthanized and peripheral blood parameters related with T2D and its commonly associated risk factors were determined. Additionally, brain cortical cytosolic homogenates and isolated mitochondrial fractions were obtained to be used in the quantification of GLP-1, insulin and glucose levels, GLP-1R protein expression levels and insulin receptor (IR) activation, by ELISA, western blotting or co-immunoprecipitation. This was followed by the colorimetric, ELISA or immunoblotting determination of several lipid and DNA oxidation, autophagic and caspase-dependent and –

independent apoptosis markers, as well as of necrotic markers. Finally, the role of Ex-4 on T2D-related brain cortical synaptic function was also evaluated by western blotting.

We observed that the continuous peripheral administration of Ex-4 was able to overcome the peripheral T2D pathological hallmarks (hyperglycemia, rate of glucose clearance from blood, HbA_{1C} levels and insulin resistance). Moreover, Ex-4 restored brain cortical GLP-1 levels in GK rats and, despite no significant effects on GLP-1R protein expression, it is possible that the drug may improve its activity in brain. However, this deserves further analysis. Surprisingly, Ex-4 did not interfere with brain cortical insulin levels or its receptor activation, suggesting that these may not be directly involved in the subsequent effects of Ex-4 in GK rat brain. Such effects include a restoration of brain glucose levels and a tendency for Ex-4 to protect against lipid and DNA oxidation. Notably, our results also pointed towards a tendentially higher activation of the autophagic process in brain cortices from the Ex-4-treated GK rats, probably as a protective strategy against the subsequently reported T2D-related caspase-3-mediated death in placebo-treated GK rats. Indeed, Ex-4 appeared to inhibit the caspase-3-induced apoptosis in T2D GK rat brain cortices, probably due (at least partially) to its tendency to retain the anti-apoptotic Bcl2 and the proapoptotic cytochrome c within mitochondria, whereas the proapoptotic Bax remained in the cytosol, ultimately leading to a slightly improved brain cortical function in Ex-4-exposed T2D rats.

Altogether, our results suggest that by ameliorating the peripheral T2D pathological hallmarks, chronic continuous subcutaneous Ex-4 has a beneficial impact in middle-aged non-obese T2D male rat brain cortex, restoring GLP-1 levels and protecting against local hyperglycemia- and oxidative stress-mediated injury, probably via the stimulation of a physiological-like autophagy and the subsequent hampering of apoptotic death and synaptic dysfunction. In sum, chronic peripheral Ex-4 administration may constitute a potential therapeutic approach against long-term complications of T2D *per se* affecting the brain.

Keywords: Type 2 diabetes, Alzheimer disease, anti-T2D drugs, incretin, GLP-1, exendin-4

Abbreviations

4-HNE	4-hydroxynonenal
8-OHdG	8-hydroxy-2'-deoxyguanosine
Aβ	Amyloid-β
ACCORD	Action to Control Cardiovascular Risk in Diabetes
AChE	Acetylcholinesterase
AD	Alzheimer disease
ADVANCE	Action in Diabetes and Vascular disease: preterAx and diamicroNmr Controlled Evaluation
AGEs	Advanced glycation endproducts
ADN	Ácido desoxirribonucleico
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
Apaf-1	Apoptosis protease-activating factor 1
APP	Amyloid precursor protein
ATG	Autophagy-related genes
ATP	adenosine trisphosphate
BACE	β-secretase
Bax	Bcl2-associated X protein
BBB	Blood brain barrier
Bcl2	B-cell lymphoma 2
BH	Bcl-2 homology
cAMP	cyclic adenosine monophosphate
CNS	Central nervous system
DISC	Death- inducing signaling complex
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
DPP-IV	Dipeptidylpeptidase-IV
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic reticulum
Epac	Exchange protein cAMP
ERK	Extracellular-signal-regulated kinases
Ex-4	Exendin-4
FADD	Fas-associated death domain
FDA	Food and Drug Administration
GABA	gamma-aminobutyric acid
GIP	Gastric inhibitory polypeptide
GK	Goto-Kakizaki
GLP-1	Glucagon-like peptide-1
GLP-1R	Glucagon-like peptide-1 (GLP-1) receptor

GLUT	Glucose transporter
GPCRs	G-protein coupled receptors
GSK-3β	Glycogen synthase kinase-3beta
GTT	Glucose tolerance test
HbA_{1C}	Glycosylated Hemoglobin A_{1C}
IDE	Insulin-degrading enzyme
IGF-1R	Insulin-like growth factor 1 (IGF-1) receptor
IR	Insulin receptor
K_{ATP}	ATP-dependent potassium channels
LAMP-2A	Lysosomal associated membrane protein-2A
LC3	Microtubule-associated protein light-chain 3
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MOMP	Mitochondrial outer membrane permeabilization
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
MVBs	Multivesicular bodies
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFTs	Neurofibrillary tangles
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PS1	Presenilin 1
RHIM	RIP homotypic interaction motif
RIP1	Receptor-interacting proteins 1
RIP3	Receptor-interacting proteins 3
ROS	Reactive oxygen species
STZ	Streptozotocin
SUs	Sulphonylureas
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
TBARS	Thiobarbituric acid reactive substances
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor (TNF) receptor
TOR	Target of rapamycin
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
TZDs	Thiazolidinediones
UPR	Unfolded protein response
VADT	Veterans Affairs Diabetes Trial
VDCC	Voltage-dependent calcium channels

CHAPTER 1

INTRODUCTION AND AIMS

1. **INTRODUCTION**

Given its ever increasing number of patients worldwide, diabetes Mellitus (DM) is nowadays a growing health care issue and a modern epidemic, with recent estimates pointing to an increase from 285 million to 439 million diabetics between 2010 and 2030 [1]. There are several types of diabetes, but the two major forms are type 1 (T1D) and type 2 diabetes (T2D), with the later accounting for more than 90% of all cases and, thus, unquestionably constituting a major public health concern [1,2].

One of the major pathological hallmarks of T2D is hyperglycemia that may arise from insulin resistance. This in turn may progress towards pancreatic β-cell failure and a generalized loss of insulin sensitivity in the later phases of the disease. Physiologically, β-cells can compensate for the loss of insulin sensitivity by other cells in the body [3]. However, and despite initially not depending on exogenous insulin administration, T2D patients may at some point need this hormone to control blood glucose levels, particularly when diet alone or treatment with oral hypoglycemic agents become inefficient [4].

Although traditionally T2D was more common in people aged above 30 years, currently its incidence is also increasing among younger people [5], probably as a result of other risk factors (besides aging) associated with modern lifestyle, namely high blood pressure, sedentarism and metabolic syndrome [6]. Interestingly, metabolic syndrome constitutes a group of disorders (*e.g.* dyslipidemia and obesity) also highly related with cardiovascular disease [1,7,8]. Besides all this, T2D also poses a huge socio-economic burden due to its commonly associated long-term complications that may lead, *e.g.*, to brain structural and functional alterations [9]. Notably, amongst such long-term deleterious influence of T2D in brain, it is included its wide implication as a risk factor for dementia (both vascular dementia and Alzheimer disease (AD)) [10], with aging constituting also a well-known common risk factor to both T2D and AD [11]. And, surprisingly, in the Mayo Clinic Alzheimer Disease Patient Registry, Janson *et al.* (2004) reported that both T2D and impaired fasting glucose were more prevalent in AD than in non-AD individuals, with 81% of AD patients exhibiting either T2D or impaired fasting glucose [12].

Several mechanisms have been hypothesized on the increased neurodegeneration and impaired cognition in T2D. For instance, it has been increasingly suggested a pivotal role for insulin resistance (and/or impaired insulin signaling), particularly upon aging [13,14]. And in line with this, AD has been increasingly recognized as a neuroendocrine-like disorder, a so-called "type 3 diabetes" [15,16] or a "brain-specific T2D" [17]. Given the close relation between both disorders, it has been also increasingly accepted that an accurate and early diagnosis of T2D, a provision of durable glycemic control and, at least in the initial phases of the disease, a convenient prevention of chronic complications (*e.g.* by the adoption of an healthy lifestyle, body weight control or moderate physical exercise) may be crucial to successfully fight against these epidemics. However, as disease progresses, some pharmacological approach (including combined therapy) may be required to successfully deal with T2D and its complications In this regard, the development of more efficient preventive anti-T2D therapies (and with less secondary effects) that also minimize any further damage to the already injured organs is of a crucial relevance to further avoid the development of long-term T2D complications, including AD.

1.1. Molecular commonalities between T2D and AD

Besides impaired insulin signaling, other abnormal biochemical mechanisms common to both T2D and AD include glucose dysmetabolism, mitochondrial dysfunction, increased oxidative stress or deposition of

amyloidogenic proteins, which, if not efficiently dealt by the cells, may ultimately lead to neurodegeneration and death [2].

Physiologically, neurons are unable to store and synthesize glucose (their main metabolite) and, thus, it has to be transported from the periphery, cross the blood-brain barrier (BBB) and target the brain. This occurs mostly via the glucose transporters (GLUTs) isoforms GLUT-1, GLUT-3 and GLUT-4 (the most abundant isoforms) [18]. However, under chronic glucose dysmetabolism (as in T2D) several brain damaging effects may occur, including the formation and accumulation of highly deleterious advanced glycation endproducts (AGEs) [19]. These AGEs arise after a sequence of events that start by the reaction between reducing sugars (*e.g.* glucose and fructose) and amino groups from proteins, yielding the end products of the *Maillard* reaction, which then become auto-oxidized and form cross-linked complexes and unstable compounds [16]. Although massive AGEs formation has been widely described in diabetic patients, they have been also detected in retinal vessels, peripheral nerves, kidneys, and central nervous system (CNS) of aged patients [20]. Importantly herein, the extent of AGEs-induced glycation of amyloid-β peptide (Aβ) being correlated with its aggregation into senile plaques, as well as with abnormally increased tau protein phosphorylation and consequently with neurofibrillary tangles (NFTs) formation [2] - the two main neuropathological hallmarks of AD [16]. Besides this, AGEs reaction with free radicals is also known to exacerbate oxidative damage and, ultimately, cellular damage [11]. In this regard, as T2D also accelerates the production of such deleterious molecules, it is not surprising that AGEs production (and eventually the vicious cycle of oxidative stress) may constitute an additional potential pivotal link between T2D and increased risk of AD.

Besides AGEs, another common mechanism to both pathologies is mitochondrial dysfunction [7]. Although mitochondria are primarily responsible for several crucial cellular processes and the main coordinators of energy metabolism (they generate over 90% of cellular adenosine trisphosphate (ATP) [2], they are also one of the major sources and targets of reactive oxygen species (ROS), which in turn are known to play a pivotal role in the pathogenesis of both AD and diabetes[21]. Thus, given mitochondria's high susceptibility to oxidative stress-mediated injury and the neurons' extreme sensitivity to alterations in their mitochondrial pool, it is plausible their dysfunction might be correlated with AD and diabetes. In this regard, our group has recently demonstrated that respiratory chain and phosphorylation systems were similarly impaired in brain mitochondria from both the 3xTgAD and chronically sucrose-treated mice (models for AD and T2D, respectively) [22]. This, together with an increased oxidative dysfunction could underlie their similar behavioral and cognitive abnormalities, including increased fear and anxiety and impaired learning and memory. Besides this, an increased brain cortical and hippocampal Aβ accumulation was reported in sucrose-treated mice, thereby point towards the development of AD features in T2D as a consequent of a metabolic dysfunction [22,23]. Importantly, these results were in line with previous reports on T2D patients predisposition for cognitive decline and eventually AD [24], as well as with other very recent studies in our group reporting an association between central insulin resistance and brain cortical and hippocampal profound mitochondrial abnormalities, and increased Aβ accumulation, tau protein hyperphosphorylation, neurodegeneration and death [25].

1.1.1. Dysfunctional mechanisms of cellular quality control in T2D and AD brains

Under physiological conditions, neuronal cells' homeostasis is maintained, *e.g.*, by protein quality-control mechanisms. Thus, in the presence of misfolded proteins, the intracellular activation of the ubiquitin-

proteosome system and/or autophagy-lysosome pathway determines the degradation or recycling of such abnormal proteins, avoiding their maintenance in the cell [26,27]. However, once these systems become dysfunctional, the accumulation and aggregation of altered proteins occur, ultimately leading to the cellular toxicity and neurodegeneration typical from, *e.g.*, AD and other neurodegenerative pathologies [28].

1.1.1.1 Autophagy

Autophagy is an evolutionarily conserved catabolic process that targets cellular organelles and other cytoplasmic constituents (as aged, dysfunctional proteins) for lysosomal recycling or degradation [29]. Several stimuli can induce autophagy, including starvation, hypoxia and chemical toxins (the most well known is rapamycin)[30] and its activation has been implicated in numerous distinct cellular processes (*e.g.* cell survival, cell death and pathogen clearance) [31]. Pathologically, strong genetic and animal data support a direct association between dysfunctional autophagy and cancer, neurodegenerative diseases and chronic inflammation [32–34].

One of the most widely used and arguably the most physiologically relevant stimulus for autophagy has been nutrient starvation [35], whose cellular detection and subsequent autophagic regulation involve nutrient-sensitive kinases, such as mammalian target of rapamycin complex 1 (mTORC1) and AMP-activated protein kinase (AMPK) [36]. AMPK is a serine/threonine kinase activated by metabolic stress or ATP consumption, leading to the activation of a wide variety of catabolic processes (as glucose uptake and metabolism), whilst several anabolic pathways (*e.g.* lipid, protein, and carbohydrate biosynthesis) are simultaneously inhibited [37]. On the other hand, target of rapamycin (TOR) is another highly evolutionarily conserved serine/threonine protein kinase that occurs from yeast to mammals (the mammalian target of rapamycin (mTOR)) [38]. Initially, TOR protein was merely identified as a target of rapamycin, but then it was also shown to regulate rapamycin-induced autophagy in yeast, even under nutrient-rich conditions [39]. Interaction between TOR proteins and their binding partners leads to the formation of at least two functionally distinct complexes in yeast: the so-called TOR complex 1 (TORC1) and TORC2, with rapamycin binding exclusively to TORC1 whereas mTORC2 is unaffected by rapamycin treatment [40]. Importantly, mTORC1 activity depends on several positive signals (such as high energy levels, normoxia, amino acids, or growth factors) to culminate in autophagic inhibition, whereas AMPK activation occurs under energy-low conditions and has been associated with autophagy induction [41].

Three types of autophagy have been identified to date: macroautophagy, microautophagy and chaperone-mediated autophagy (Fig. 1.1). In literature, the term "autophagy" usually corresponds to macroautophagy, which is characterized by the formation of double membrane vesicles – the autophagosomes [35]. Macroautophagy was first genetically characterized in yeast, yielding to the identification of a family of autophagy-related genes (ATG) that function as its direct executioners [42]. More specifically, the yeast ATG8 and its mammalian homologue microtubule-associated protein light-chain 3 (LC3) are key autophagic-related proteins, which are recruited to the autophagosomal double-membranes [43], thereby monitoring the mammalian autophagosomal formation for subsequent sequestration of material to be delivered to lysosomes [29]. Besides LC3, beclin-1 is another pivotal protein on the autophagic process, being a mammalian orthologue of yeast Atg6 highly involved in the vesicle formation during autophagy [44] and actively cooperating with the phosphoinositide 3-kinase (PI3K) pathway to enhance the formation of the autophagic vacuoles [29]. Hence, beclin-1 has been also commonly used to monitor autophagic activity.

In microautophagy, lysossomes engulf small cytosolic components through invagination of the

lysossomal membrane directly into its lumen [45]. This dynamic reportedly resembles the formation of multivesicular bodies (MVBs) in the late endosome [46]. On the other hand, during chaperone-mediated autophagy, a selective lysosomal pathway for the degradation of cytosolic proteins, these are directly translocated into lysossomes by means of lysosomal associated membrane protein-2A (LAMP-2A) [47]. This process is a secondary response to starvation in mammals and does not involve any membrane reorganization [48].

Studies from our group on the relation between brain mitochondrial dysfunction upon DM and/or AD and autophagy provided evidence that, upon insulin signaling-mediated activation of autophagy, the subsequent degradation of aged and dysfunctional proteins was accompanied by an increase in the aminoacid pool, which could provide the energy necessary for the synthesis of new proteins [49,50]. This hypothesis was further supported by a recent study involving streptozotocin (STZ)–induced T1D rats treated with insulin [51]. In insulin-treated T1D rats, lower LC3-II levels compared to non-treated diabetic animals were accompanied by a decrease in tau protein phosphorylation at the serine 396 residue [51], suggesting that a decrement in autophagy activation in the insulin-treated animals could be related with a stimulation of calpain-mediated tau degradation and, ultimately, with a decrement in tau protein phosphorylation [52].

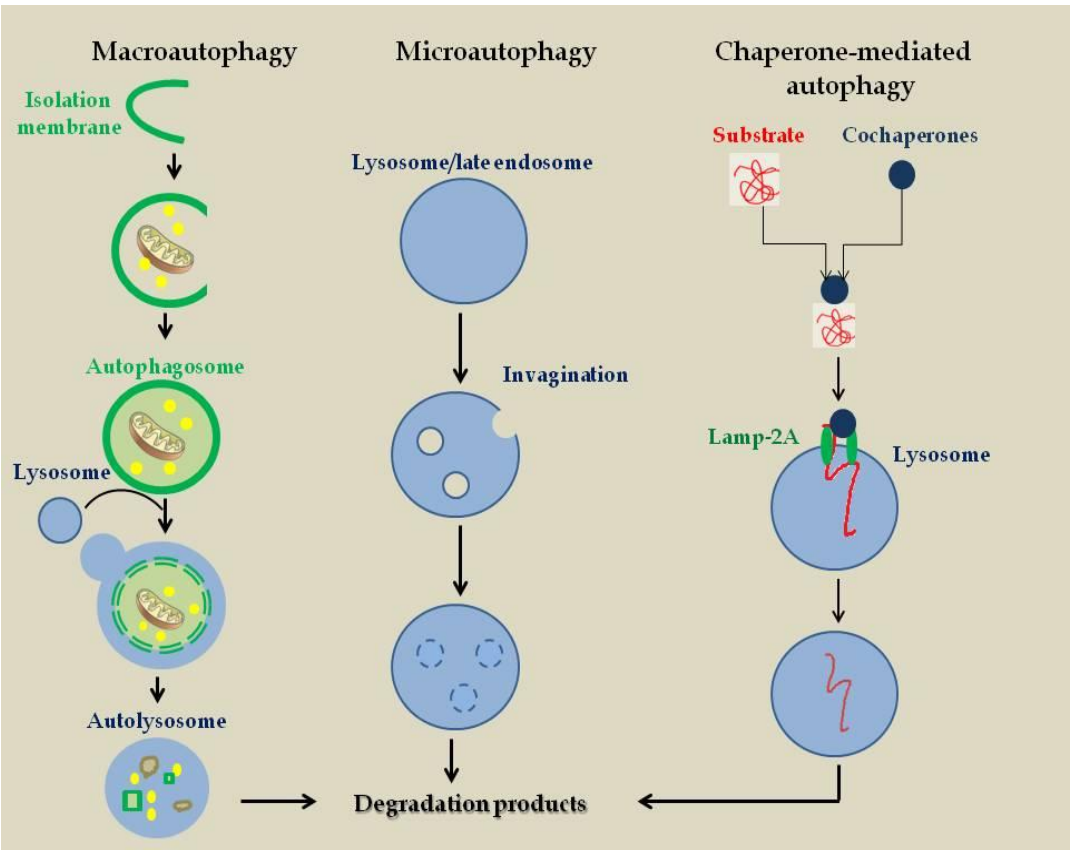


Figure 1.1. ***Different types of autophagy***. In macroautophagy, a portion of cytoplasm (including organelles) is enclosed by an isolation membrane (the phagophore) to form an autophagosome, whose outer membrane then fuses with the lysosome and allows for degradation of its content in the resulting autolysosome. In microautophagy, small pieces of the cytoplasm are directly engulfed by inward invagination of the lysosomal or late endosomal membrane for subsequent degradation. Chaperone-mediated autophagy involves the recognition of substrate proteins and co-chaperones. Then, they bind with lysosomal LAMP-2A, being translocated into the lysosomal lumen. Regardless the type of autophagy activated, the resulting degradation products can be used for, *e.g.*, new protein synthesis, energy production or gluconeogenesis. [Adapted from Mizushima and Komatsu, 2011].

Despite the extensive literature, the precise role of autophagy in AD is still not fully understood. *In vitro* studies reported that under nutrient deprivation, treatment with $A\beta_{25-35}$ and $A\beta_{1-42}$ induced autophagy in a SH-SY5Y cell line overexpressing EGFP-LC3 [53]. Moreover, Hung *et al.* (2009) demonstrated that using the ATG7 siRNA to inhibit the formation of autophagosomes early in the process significantly enhanced Aβ-induced

neurotoxicity [53]. Additionally, neuronal exposure to Aβ promoted autophagosomal formation and protected against the development of apoptotic characteristics, whereas the inhibition of autophagy exacerbated neuronal apoptosis [54]. Furthermore, lysosomal autophagy has been also reported to play a role in the clearance of tau protein (which when dysfunctional, results in the formation of tau oligomers) [55]. Overall, these observations led to the hypothesis that autophagy could have a neuroprotective role against Aβ-induced neurotoxicity [50]. Nevertheless, others reported that autophagy could contribute to the degeneration of dystrophic neurites. In line with this, Yu *et al.* (2004, 2005) [30,56] showed that autophagic vesicles contain amyloid precursor protein (APP) and a high content of β-secretase (BACE), necessary to generate Aβ. This was supported by the detection of both $A\beta_{1-40}$ and $A\beta_{1-42}$ in purified autophagic vesicles from transgenic mice overexpressing APP [30,56]. Accordingly, it was demonstrated that the AD-induced impairment in the maturation of autophagosomes and in their retrograde transport towards the neuronal soma leads to a large accumulation of autophagic vesicles along dystrophic and degenerating neurites, which could contribute to the local accumulation of Aβ within plaques [57]. Moreover, the functional disruption in the endocytic pathway, together with an overexpression of lysosomal system components in AD may exacerbate Aβ overproduction and consequent neuronal death [58]. Importantly herein, it was recently found that the brains of patients with advanced AD had a reduced expression of beclin-1, which was consistent with reports on the decreased neuronal autophagy in a transgenic mouse model of AD with deletion of beclin-1, as well as with an increased accumulation and deposition of intraneuronal Aβ [59]. Consistently, upon mTOR inhibition with rapamycin and the subsequent signaling restoration, cognitive deficits were rescued and Aβ and tau levels improved, probably due to increased activation of autophagy [49].

From the above, most evidences appear to support a neuroprotective role for autophagy in the early stages of AD pathology, whilst as disease progresses autophagy will probably potentiate neuronal degeneration and death.

1.1.1.2. Main cell death mechanisms in T2D and AD brain: apoptosis and necrosis

a) Apoptosis

Under physiological conditions, apoptosis is a crucial biological process throughout the organism, but most particularly in the nervous system, whereby it regulates the development and tissue homeostasis. However, apoptosis deregulation may lead to several pathological alterations, including developmental defects, autoimmune diseases or cancer [60]. During brain development, cell death is essential for the regulation of neuronal cell number and to protect against the propagation of damaged cells, whereas its deregulation in the adult nervous system has been implicated in neurodegeneration and neurodegenerative disorders [61].

Morphologically, apoptosis is characterized by cell membrane blebbing, cell shrinkage and chromatin condensation, followed by DNA fragmentation, engulfment by macrophages or other neighboring cells (to avoid an inflammatory response in surrounding tissues) [62]. Additionally, apoptosis may be accompanied by phosphatidylserine exposure on the outer leaflet of the plasma membrane, as well as by changes in mitochondrial membrane permeability, with the subsequent release of mitochondrial proteins (*e.g.* cytochrome c) from the intermembrane space [63].

There are two main apoptotic pathways: the extrinsic (or death receptor pathway) and the intrinsic pathways (also known as the mitochondrial pathway) [64]:

- The extrinsic pathway - can be initiated by numerous external stimuli, including binding of death-inducing

ligands to cell surface receptors [65]. This cascade usually involves cell surface death receptors (namely Fas, tumor necrosis factor (TNF) receptor (TNFR), or TRAIL receptors) that oligomerize upon death ligand stimulation, thereby recruiting the adaptor protein Fas-associated death domain (FADD) and caspase-8 to form a death-inducing signaling complex (DISC). Autoactivation of caspase-8 at the DISC then activates downstream effector caspases (including caspases-3, -6 and -7) to further continue the cell death program [66].

- The intrinsic pathway – can be initiated by intrinsic signals (such as irradiation- or chemically-induced DNA damage, growth factor deprivation or oxidative stress) that ultimately may involve mitochondria, with the release of mitochondrial cytochrome c into the cytoplasm and the subsequent induction of a caspase cascade. More specifically, once in the cytosol cytochrome c binds to the apoptosis protease-activating factor 1 (Apaf-1) and procaspase-9, generating an intracellular DISC-like complex known as "apoptosome", where caspase-9 is activated and subsequently processes caspase-3 [67].

Importantly, we must bear in mind that both extrinsic/death receptor and intrinsic/mitochondrial apoptotic pathways may ultimately converge on caspase-3 to drive the terminal events of programmed cell death [60].

Regarding caspases, these apoptotic executioners are cysteine proteases that use the sulfur atom in cysteine to cleave polypeptide chains. These enzymes search for a specific sequence in their target proteins, cleaving their substrates typically on the carboxyl side of aspartate amino acids (thus the name "caspase": "c" for cysteine protease and "asp" for the strong aspartate preference) [68,69]. According to the specific substrates cleaved by each caspase during apoptosis progression, typical biochemical and morphological changes arise, thus rendering the detection of activated caspases a noteworthy marker of this type of cell death [70]. Traditionally, initiator caspases were considered to be autoproteolytically activated when closely to each other – this was called the *induced proximity model* [66]. Later on, this model was refined to the *proximity induced dimerization model*, according to which the apoptosome promotes caspase-9 homodimerization due to its increased local concentration [71]. Likewise, DISC induces dimerization and consequent auto-activation of caspase-8 [72]. Accordingly, activated initiator caspases will normally cleave and activate effector procaspases, which then cleave several downstream death substrates to induce cell death [73]. Accordingly, several lines of evidence suggest that the three downstream effector caspases (caspases-3, -6 and -7) are usually more abundant and active than the upstream "initiator" caspases-8 or -9 [74]. Nevertheless, genetic evidence has shown that loss of caspase-3 may also result in gross brain malformations and premature death [75], with caspase-3 knockout mice also displaying a defective response to both intrinsic and extrinsic apoptotic pathways stimuli [76]. Thus, caspase-3 has been recognized as the crucial executioner caspase [60].

From the above, we can infer that mitochondria is a central regulator of the intrinsic apoptotic pathway, with mitochondrial outer membrane permeabilization (MOMP) playing a crucial role herein [77]. Importantly, MOMP mediation and control is known to involve the Bcl-2 family members, which in turn are also key regulators of apoptosis [78]. Bcl-2 family of proteins involves both pro- and antiapoptotic proteins that share sequence homology in Bcl-2 homology (BH) domains [79]. Regarding the antiapoptotic group of proteins (as Bcl-2 and Bcl-xL), their homology occurs in four BH domains (BH1 to 4), whereas the proapoptotic ones can in turn be subdivided into the "multidomain" and the "BH3-only" subfamilies [80]. The multidomain proapoptotic subclass includes Bax and Bak, that display homology in BH domains 1-3, while the BH3-only proteins (*e.g.* Bid and Bim) although structurally similar to multidomain family members, their sequence similarity is limited to the BH3 domain [80].

Under physiological conditions, Bax proteins reside in the cytosol and do not dimerize with each other or with other Bcl-2 family members. However, upon the induction of apoptosis, Bax proteins acquire an active conformation and translocate into mitochondrial and endoplasmic reticulum (ER) membranes, thereby forming homo-oligomers or heterodimerizing with other Bcl-2 family members, resulting, *e.g.*, in Bax/Bcl-2 heterodimers [81].

In 1999, Russel *et al.* observed that hyperglycemia, a hallmark of T2D, induced neurite degeneration and several apoptotic features (including activation of caspase-3) in rat dorsal root ganglion neurons [82]. These results were correlated with the observation, in an *in vivo* hyperglycemic model, of nuclear and cytoplasmatic apoptotic changes on top of disruption of mitochondria functionality in dorsal root ganglion neurons and Schwann cells [82].

As previously referred, AD is the best known form of dementia, characterized by an impaired cognition that has been strongly correlated with synaptic degeneration and neuronal death in the hippocampus, amygdala and associated regions of the cerebral cortex, probably as the result of Aβ peptide-enriched amyloid plaque formation [83]. But the most striking herein was that, besides these classic Aβ deposits, neurons from AD patients also displayed an increased caspase activity, damaged DNA and alterations in the expression of Bcl-2 family members [84], pointing towards an apoptotic-mediated neuronal death. These observations were further supported by the induction of apoptosis upon exposure of cultured neurons to Aβ, in a process involving increased oxidative stress and/or expression of Fas ligand in neurons and glia [85]. Alternatively, microglia can be activated, promoting TNFα secretion and activation of the TNFR-mediated extrinsic apoptotic pathway [86,87]. Importantly, the use of antioxidants intended to suppress lipid peroxidation and compounds known to stabilize calcium homeostasis were shown to protect neurons against Aβ-induced apoptosis [88]. Additional evidences demonstrating the involvement of apoptotic cascades in AD pathology also include the observation that APP may function as a substrate for caspase-3 [89].

It deserves to mention that an increasingly proposed alternative, crucial subcellular regulator of apoptosis (particularly in AD) is endoplasmic reticulum (ER) [78]. This organelle's role in apoptosis propably involves mitochondria sensitization to a variety of extrinsic and intrinsic death stimuli and/or its own initiating cell death signals [90]. Interestingly, several evidences showed the direction of proteins from all Bcl-2 classes towards the ER [78] and although the underlying mechanisms are still poorly understood, it is plausible that ER stress-associated apoptosis may involve disturbed glycosylation, misfolded proteins (as in glucose deprivation impairment in unfolded protein response), cytoplasmic death pathways induced by the mobilization of ER calcium stores, sensitization of mitochondria to respond to direct proapoptotic stimuli [65]. This in turn would lead to caspase-12 activation and possible direct processing of downstream cytosolic caspases [91]. Despite some controversy, authors suggested that active caspase-12 was able to cleave and activate procaspase-9 directly, then resulting in downstream caspase-3 activation [92,93]. Although caspase-12 is a ubiquitously expressed enzyme, being synthesized as an inactive procaspase composed by a regulatory prodomain and two catalytic p20 and p10 subunits [94], its unique feature is its almost exclusive activation by insults that bring forth ER stress [92]. Therefore, caspase-12 activation rendered it a useful marker for ER stress-related apoptosis. After ER stress, this organelle tries to reestablish homeostasis by activating the unfolded protein response (UPR), aiming at protecting and triggering adaptive pathways [95]. Nonetheless, chronically activated UPR may also have negative effects on cell survival, as it affects the folding and trafficking of proteins and has been suggested to play a role in neurodegenerative diseases, including AD [96]. Indeed, it has been hypothesized that ER stress may interfere with

the trafficking and processing of APP during the secretory pathway, leading to an intracellular overproduction of Aβ and consequent oxidative stress that may culminate in apoptotic cell death in AD [97].

b) Necrosis

Another type of cell death that has been increasingly described is necrosis. Together with apoptosis and autophagy, necrosis contributes for the maintenance of homeostasis in multicellular organisms, by keeping the balance between cell proliferation and cell death [63]. Necrosis is a highly regulated and programmed event, sharing a variety of components with other types of cell death [98]. Morphologically, necrosis is characterized by a rapid cytoplasmic swelling that culminates in plasma membrane rupture and organelle breakdown [99]. Traditionally, necrosis was faced as a consequence of severe physical and/or chemical stress factors (*e.g.* heat, osmotic shock, mechanical stress, freeze/thawing and high concentrations of hydrogen peroxide) [63]. Molecularly, receptor-interacting proteins 1 and 3 (RIP1 and RIP3, respectively) seem to be critical inducers of programmed necrosis (Fig. 1.2).

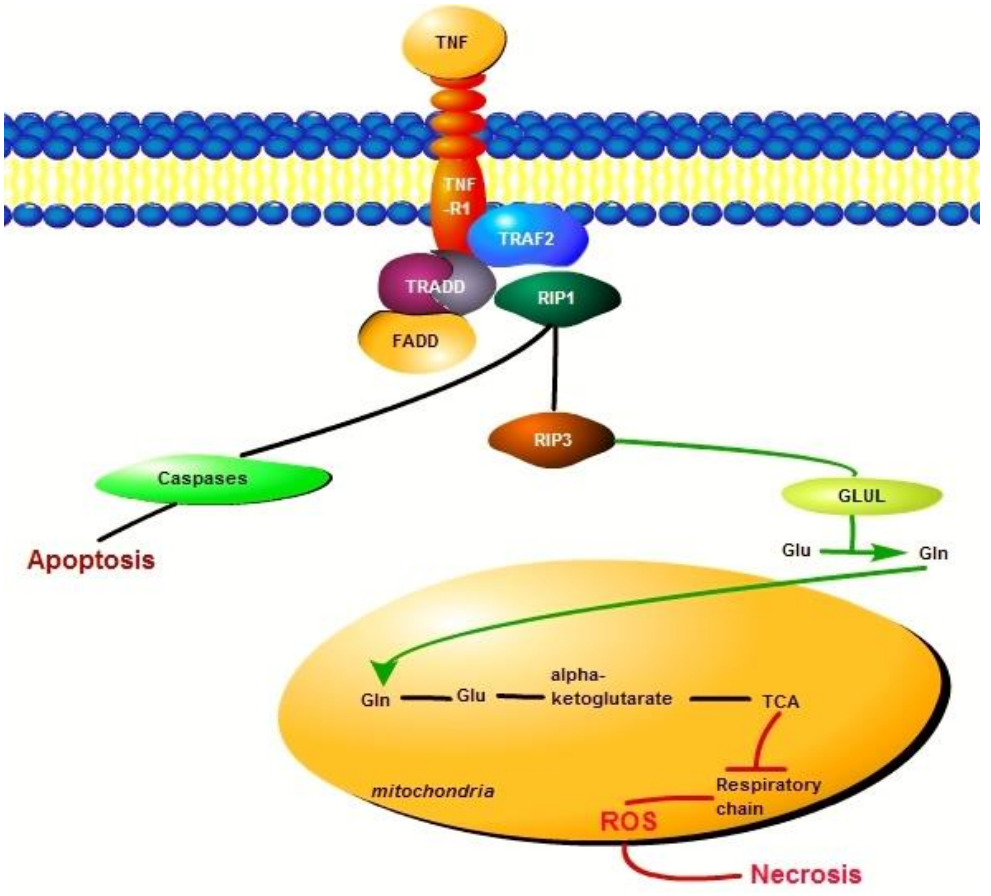


Figure 1.2. ***Potential mechanisms of RIP3/RIP1-mediated necrosis***. In cells depleted from RIP3, TNF induces apoptosis independently from this protein; however, when RIP3 is sufficiently expressed, TNF stimulation opens the gateways to use glutamate (Glu) or glutamine (Gln). As a consequence of such abnormal metabolic stimulation, reactive oxygen species (ROS) levels may rise, mostly due to the mitochondrial respiration chain, initiating the alterations typical from necrosis cell death. Abbreviations: FADD, Fas-associated death domain; GLUL, glutamate ammonia ligase; RIP, receptor-interacting protein; ROS, reactive oxygen species; TCA, tricarboxylic acid cycle; TNF, tumor-necrosis factor; TNF-R1, tumor-necrosis factor receptor 1; TRADD, TNF-receptor-associated death domain; TRAF, TNF-receptor-associated factor. [Adapted from Zhang *et al*, 2010].

These proteins belong to the RIP kinase family, whose members have different functional domains, despite sharing a homologous kinase domain [100]. More specifically, RIP1 contains a C-terminal death domain that allows it to be recruited to various complexes, which will initiate different signaling pathways [101]. Conversely, RIP3 has a C-terminus that is unique among all known protein domains. Besides this, it was also

found a RIP homotypic interaction motif (RHIM) in the intermediate domain of RIP1 and in the C-terminus of RIP3, that appears to be necessary for the interaction between both proteins [102]. Interestingly, RIP1 is known to be also involved in several signaling pathways and play other numerous roles than necrosis, being referred to as a key switch of cell fate regulation [103], as it is able to direct the cell to an apoptotic cell death instead of a necrotic one [104]. Additionally, RIP1 mediates the activation of the prosurvival transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) [105]. On the other hand, Cho *et al.* (2009) reported that RIP1 phosphorylation upon necrosis was regulated by RIP3, suggesting that the later one may be the central controller of programmed necrosis, by initiating the pronecrotic kinase cascade [105]. In the brain, RIP1 and RIP3 have been implicated as part of the necrosome complex that leads to programmed necrosis after neonatal hypoxic injury [98]. Additionally, it has been suggested that the loss of hypoccampal neurons occurring during the initiation and progression phases of AD could also involve cytokine-driven neuroinflammation and neurotoxicity, whereby TNFα appeared to be a key proinfammatory cytokine [106]. In line with this, RIP3-mediated necroptosis (regulated necrosis) was shown to be activated in mouse hippocampus after intracerebroventricular injection of TNF-α, whilst RIP3 deficiency attenuated the loss of hippocampal neurons due to TNF-α [107].

1.1.2. Endogenous insulin/insulin receptor signaling as the missing link between T2D and AD

1.1.2.1. The role of endogenous insulin in normal brain

Traditionally, insulin was recognized as a crucial regulator of peripheral glucose metabolism, particularly in fat, liver or muscles. However, recent evidences showed that, besides reaching high levels in CNS, the hormone is also able to control several brain functions [108]. These include cognitive function, memory, learning and synaptic plasticity, most likely via the activation of insulin/insulin receptor (IR) signaling pathways [109]. Regarding its origin, it is believed that most CNS insulin derives primarily from pancreatic β-cells, being transported mostly across the BBB [110]. Additionally, some of the peripherally-synthesized insulin may also directly diffuse into the CNS via a circumventricular region with a “leaky” BBB - the area postrema [111]. However, some striking data also point towards the *de novo* brain insulin synthesis, particularly in pyramidal neurons (*e.g.*, from hippocampus, prefrontal cortex and olfactory bulb, but not glial cells), being then exocytotically released [17]. Once in CNS, insulin rapidly binds to its highly abundant and ubiquitously distributed specific receptor (with a particular emphasis on olfactory bulb, hypothalamus, cerebral cortex, cerebellum, hippocampus and striatum)[112], initiating its signal transduction [110].

1.1.2.2. Brain insulin/IR dysfunction in T2D and AD

Several lines of evidence suggest that the T2D-associated impairment in brain insulin uptake may deprive this tissue from the peptide’s beneficial effects [108]. In this perspective, whilst acutely increased peripheral insulin levels may transiently increase its levels in cerebrospinal fluid, the insulin resistance- or T2D-related peripheral hyperinsulinemia may chronically downregulate IRs at BBB, impairing brain insulin uptake [110] and ultimately affecting learning, memory and cognition [112–114]. Therefore, it has been increasingly proposed that dysfunctional insulin/IR-mediated signaling might constitute the missing link between diabetes (particularly T2D) and AD [115]. This has been further supported by the hormone’s role in the regulation of Aβ

deposition and tau hyperphosphorylation (the main neuropathological hallmarks of AD) [116]. More specifically, insulin/IR dysfunction has been associated with abnormal tau protein hyperphosphorylation, reduced APP processing and impaired Aβ release for extraneuronal clearance, thereby promoting the abnormal Aβ accumulation inside neurons [110]. Additionally, under hyperinsulinemic conditions, the insulin in excess competes with Aβ for insulin-degrading enzyme (IDE, a metalloprotease that degrades both insulin and Aβ), resulting in abnormal Aβ accumulation and formation of senile plaques [117]. Besides this, Ho *et al.* (2004) [118] observed that a diet-induced insulin resistance and a hyperinsulinemic state in an AD-like model decreased IDE levels and Aβ accumulation, whilst rosiglitazone (an insulin sensitizer) administration reduced $A\beta_{1-42}$ levels (the main pathological form of Aβ in brain tissues) and improved learning and memory[119]. These results suggested that the restoration of insulin signaling (as with rosiglitazone) and the subsequent decrement in the hormone available could limit its competition with Aβ, thus allowing the peptide degradation by IDE [119].

Interestingly, previous studies in our group showed that insulin may exert a neuroprotective role against oxidative stress [120,121]. Indeed, exogenously added insulin was able to activate neuronal IR/IGF-1 receptor (IGF-1R)-mediated signaling pathways upon oxidative stress, thereby promoting PI3K- and extracellular-signal-regulated kinases (ERK) 1/2-mediated signaling and inhibiting glycogen synthase kinase-3beta (GSK-3β). As a result, neurons were protected against neuronal lipid and protein oxidation, particularly against the formation of 4-hydroxynonenal (4-HNE, a byproduct of oxidation) adducts on neuronal GLUT3 glucose transporters [120]. This was accompanied by a stimulation of neuronal glucose uptake and downstream metabolization into pyruvate, thereby leading to an overall increase in neuronal energy levels, preventing both necrotic and apoptotic neuronal death and restoring neuronal viability [122]. These results were in accordance with the insulin-mediated activation of mitogen-activated protein kinase (MAPK) signaling (namely p38 MAPK) and the subsequent suppression of caspase-3 activity, that besides protecting against neuronal apoptosis could also play a role in memory and learning [109]. Interestingly, we also reported that, under T2D and/or oxidative stress, insulin was able to modulate the transport of the amino acid neurotransmitters γ-aminobutyric acid (GABA) and glutamate[121,123,124].

Besides insulin, IGF-1 and -2 and relaxin also share structural similarities and belong to the same protein family [125]. Importantly, IGF-1 also crosses the BBB and is ubiquitously distributed in rodent and human brains, having several pivotal effects in CNS [126,127]. Therefore, it is not surprising that, similarly to insulin/IR, IGF-1/IGF-1R-mediated signaling also regulate brain glucose metabolism, neuronal growth and differentiation, neuromodulation, synaptic transmission, memory/learning and neuroprotection [17]. Accordingly, several studies reported that impaired insulin and IGF-1 signaling in T2D models were accompanied by neuronal loss, neurite degeneration, APP dysmetabolism and tau protein hyperphosphorylation [126,128].

1.2. Could anti-T2D therapies be also promising against AD?

1.2.1. The main therapeutic goals in T2D management

From the above, it seems unquestionable that T2D and AD are two intrinsically related pathologies with several common mechanisms. Therefore, it has been increasingly proposed that an anti-T2D treatment could be also beneficial against AD (Fig. 1.3) and, thus, the use of anti-T2D drugs (already in clinical use or under clinical trials) in the context of AD has been deeply and increasingly explored in the recent years [129].

Regarding T2D and its chronic complications' management, it is widely accepted that, at least in the initial phases of the disease, maintenance of blood glucose levels within the normal range constitutes a highly efficient approach to reduce the risk of such long-term vascular and cardiovascular complications. Nevertheless, as T2D progresses, this first approach may lose efficiency and then its successful management may also include the control of blood pressure and lipid levels [130]. Thus, we must bear in mind that, although the main goal of diabetes therapy relies on an optimal glycemic control (Fig. 1.3), in the long run this may not be enough to reduce the cardiovascular risk [131]. Additionally, recent outcomes from clinical trials (as the Action in Diabetes and Vascular disease: preterAx and diamicroNmr Controlled Evaluation (ADVANCE), Action to Control Cardiovascular Risk in Diabetes (ACCORD) and Veterans Affairs Diabetes Trial (VADT)) led to a new guideline on the consideration of specific patient/disease factors when prescribing a patient-adjusted therapeutic strategy against hyperglycemia [132].

Moreover, besides the achievement of euglycemia, efficient T2D therapeutic approaches should also aim at decreasing glycosylated hemoglogin A_{1C} (HbA_{1C}) (Fig. 1.3) [133], whose levels provide an indirect estimation on the average plasma glucose levels in the last 2-3 months, thus giving an idea on the "long- term" (rather than "acute") control of blood glucose levels [134]. However, some controversy still remains on the ideal HbA_{1C} levels. Importantly, several studies reported that lowering HbA_{1C} levels by 1% reduced microvascular complications by ~30%, as well as the risk for myocardial infarction and death from any cause after 10 years of intensive glucose control in newly diagnosed T2D patients [135]. In this perspective, the guidelines suggest that an appropriate anti-T2D therapeutic should also consider the initial values of HbA_{1C} [136]. However, as most oral anti-hyperglycemic agents can reduce HbA_{1C} by 1.5-2.0% from baseline levels of 8.5- 9.5%, this means that a patient with a baseline HbA_{1C} level higher than 9% will probably experience some difficulties in achieving a therapeutic goal of less than 7.0% and, therefore, may require a combination therapy in a near future [131].

It is also well accepted that T2D treatment should also aim at the reduction in insulin resistance (*e.g.* with diet, exercise, and/or drug therapy) and/or the stimulation in insulin secretion (Fig. 1.3)[137]. Therefore, it is not surprising that, in the later stages of disease, T2D patients may also need insulin injections [9]. However, as discussed later, these may have the inconvenient of repeated insulin-induced hypoglycemic episodes and, for this reason, several promising drugs are being tested to better address T2D pathogenesis and to overcome the risk for hypoglycemia and/or other associated risk factors (as obesity). In this perspective, as obesity is one of the risk factors closely related with T2D progression and the development of late complications, guidelines also refer that patient's body weight should be also taken into account in the chosen therapeutic strategy [138]. Notably, this might be even more relevant given that body weight gain is a frequent secondary effect of some anti-diabetes therapies [138]. For this reason, recently developed drugs also have into account their effects on adipogenesis and fat mass regulation [139].

From the several classes of anti-T2D compounds already in clinical use (*e.g.* biguanides, sulphonylureas, thiazolidinediones), we will next give an overview on the pros and cons of insulin and incretin-based agents (especially exendin-4) as potential therapeutic approaches against AD.

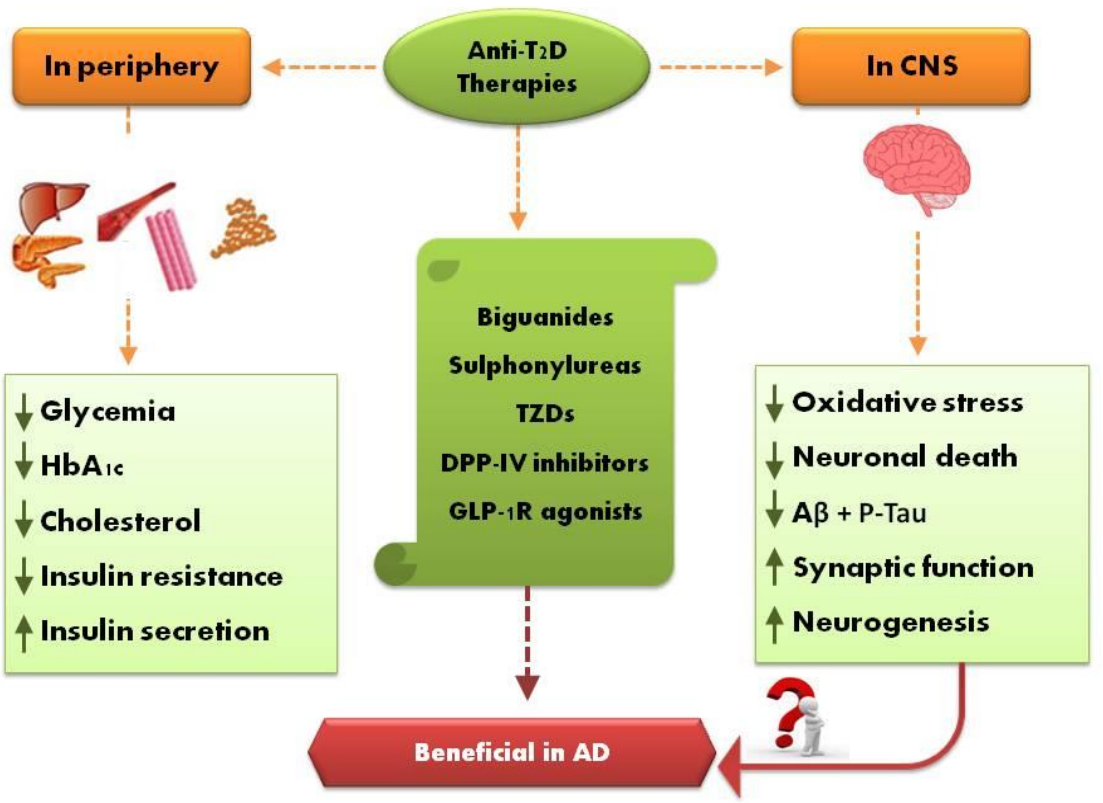


Figure 1.3. ***Effects of several anti-diabetic agents in the crosstalk between peripheral system and CNS***. Some of available anti-T2D classes of drugs include biguanides, sulphonylureas (SUs), thiazolidinediones (TZDs) and the more recent incretin-based therapies, dipeptidylpeptidase-IV (DPP-IV) inhibitors and glucagon-like peptide-1 (GLP-1) receptor (GLP-1R) agonists. Some of the main goals in T2D management include the decrement in hyperglycemia, HbA_{1C} and total cholesterol levels. Additionally, both the reduction in insulin resistance and restoration of insulin secretion have been increasingly faced as a highly relevant goal. This might be even more crucial given that insulin/IR signaling dysfunction is a common feature to both T2D and AD. Therefore, anti-T2D therapies may also benefit the CNS by, *e.g.*, counteracting oxidative stress, mitochondrial dysfunction and neuronal death, and/or by stimulation of neurogenesis and synaptic function. Noteworthy, some of these anti-T2D compounds were also reported to reduce Aβ accumulation and tau protein hyperphosphorylation, thus suggesting a potential beneficial impact against AD [Adapted from Sebastião *et al.* (unpublished)].

1.2.2. Exogenously-administered insulin as an anti-T2D and anti-AD therapy: the pros and cons

Traditionally, peripherally-administered insulin has been used as an anti-T1D therapy, aiming at controlling blood glucose levels and preventing its chronic complications [132,140]. But, as previously mentioned, it has been also increasingly used in T2D patients, particularly in the later stages of disease, when anti-hyperglycemic agents are no longer efficient. However, the frequent hypoglycemia episodes occurring after insulin therapy [131] have been also increasingly described to negatively affect the hippocampus, external cortical layers and striatum, leading to neuronal death and subsequent memory, learning and other neurological deficits, seizures and eventually coma [141,142]. In this perspective, our group has recently demonstrated that insulin-induced hypoglycemia leads to brain mitochondrial dysfunction and increased oxidative stress [22,143]. Moreover, plasma aspartate, glutamate, glutamine and taurine levels increased, whereas GABA levels decreased after an acute episode of insulin-induced hypoglycemia in rats [140]. Under similar conditions, rat brain cortical synaptosomes displayed higher glutamate and taurine levels, while GABA was decreased [140]. Additionally, the release of the excitatory amino acids aspartate, glutamate and taurine from hypoglycemic synaptosomes was further exacerbated by depolarization, thereby suggesting a possible mechanism by which an additional

metabolic insult may underlie the neuronal injury and degeneration, as well as the cognitive impairment often associated with diabetes [140].

Despite these limitations posed by exogenous insulin therapy, we must be aware that, as previously described, insulin (particularly the endogenous one) has been also widely recognized as a crucial hormone for the CNS. And given the ubiquitous localization of IRs in hippocampus, and entorhinal and frontal cortices, together with its expression primarily at the synapses, it is not surprising that physiological IR-mediated signaling may contribute to synaptic remodeling and memory formation [144]. Furthermore, insulin's benefits in brain also include the modulation of Aβ formation/clearance, thereby protecting against its detrimental effects on synapses [128], as detailed in section *1.1.2.*

1.2.3. Incretin-based anti-T2D drugs: GLP-1 receptor agonists and DPP-IV inhibitors

Incretins were discovered in the 1960s, after the increase in insulin secretion induced by a glucose load [130,145,146]. This effect was due to the simultaneous action of two gastrointestinal hormones: GLP-1 (an incretin of 30 amino acid length, secreted by the enteroendocrine K cells) and glucose-dependent insulinotropic polypeptide (or gastric inhibitory polypeptide (GIP), secreted by the enteroendocrine L cells) [130,146]. More recently, it has been shown that the two hormones combined not only account for about 70% of the insulin response to glucose [1], but they also stimulate insulin synthesis and secretion [146]. The earliest endocrine metabolism activated after a meal is the insulinotropic incretin effect that consists on the activation of the potent insulinotropic GLP-1 immediately after GIP secretion and despite the controversy on the underlying mechanism, it is possible that both central and peripheral actions of GLP-1 may stimulate insulin and inhibit glucagon secretion to restore the normal blood glucose levels [1,146,147] reduce gastric emptying, appetite, food intake and body weight [132,146,148–150]. Together with these peripheral effects, both GIP and GLP-1 can bind and activate structurally distinct G-protein coupled receptors (GPCRs) [111], with GIP receptor being predominantly expressed on islet β-cells and (to a lesser extent) in CNS and in adipose tissue, whereas GLP-1 receptor (GLP-1R) is expressed in islet α and β cells, central and peripheral nervous systems, heart, kidney, lung, and gastrointestinal tract [111]. Physiological activation of GLP-1R-mediated aerobic metabolization of glucose via glycolysis and the subsequent rising in cytosolic ATP levels have been described to close the hyperpolarizing ATP-dependent potassium (K_{ATP}) channels, depolarizing pancreatic β-cells and allowing calcium influx through voltage-dependent calcium channels (VDCC), with the concomitant calcium-dependent insulin exocytosis [148,149,151] (Fig. 1.4). However, insulin secretion may be also stimulated by other intracellular signalling cascades involving, *e.g.*, cAMP or its downstream target Epac (Exchange protein cAMP), protein kinase A (PKA), AMPK, protein kinase C (PKC) and MAPK [111,131,146,151,152]. Once blood glucose levels return to normal, GLP-1-induced insulin exocytosis is decreased [131,146] (Fig. 1.4).

Notably, several lines of evidence point towards a simultaneous CNS-mediated intervention on peripheral GLP-1-induced glucoregulation [1,146]. And, as discussed below (*Section 1.2.4*), an increasing body of literature has been suggesting that anti-T2D drugs from the classes of GLP-1 analogues and dipeptidylpeptidase-IV (DPP-IV) inhibitors may also constitute promising therapeutic strategies against chronic T2D complications affecting the CNS, particularly dementia and AD.

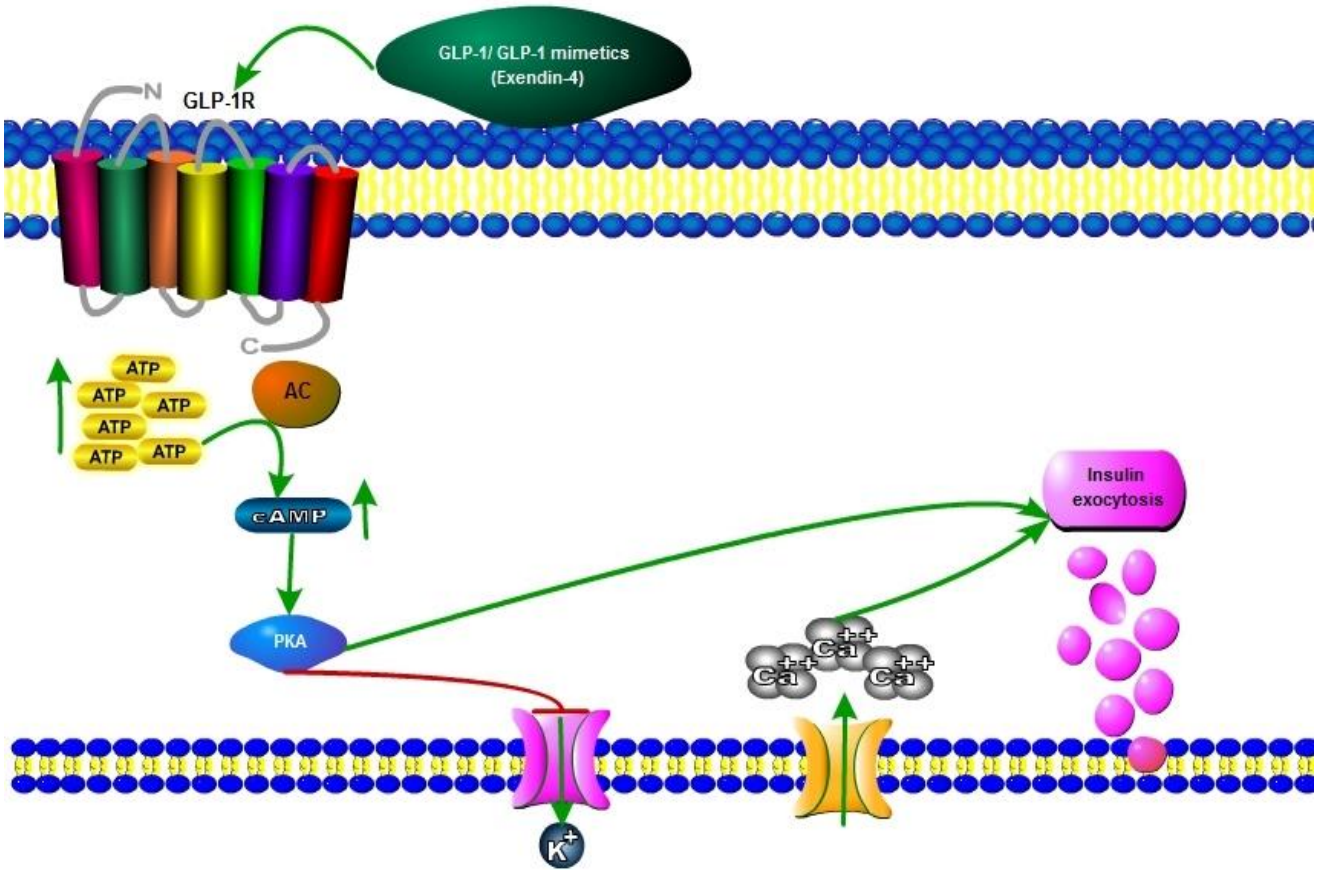


Figure 1.4. ***Mechanisms of first phase of insulin secretion by GLP-1 mimetics. GLP-1 and GLP-1R agonists exert their actions by binding to GLP-1R, a G-protein coupled receptor (GPCR).*** GLP-1R is ubiquitously expressed throughout the whole body, including peripheral and central nervous systems. Despite some controversy, it has been hypothesized that incretin receptor activation in β-cells after a meal leads to glucose metabolization through glycolysis and the subsequent increase in cytosolic ATP content. Then, the hyperpolarizing K_{ATP} channels close and β-cell membrane depolarizes, allowing calcium influx to occur via the voltage-dependent calcium channels (VDCC) and culminating in calcium-dependent insulin exocytosis. Alternatively, insulin secretion may be also stimulated by intracellular signalling cascades involving, *e.g.*, cyclic AMP (cAMP) or its downstream targets Epac (Exchange protein activated by cAMP), protein kinase A (PKA), AMP kinase (AMPK), protein kinase C (PKC) or MAPK. As blood glucose levels return to normal, GLP-1-induced insulin exocytosis is decreased. [Adapted from Sebastião *et al.* (unpublished)].

Despite such apparent efficiency, due to its almost complete degradation by DPP-IV (an ubiquituously expressed aminopeptidase that occurs, *e.g.*, in liver, lung, kidney, endothelium and lymphocytes)[153], human endogenous GLP-1 actions are highly limited due to its very short half-life (1-2 minutes) [154]. And, as this rendered the therapeutic use of the naturally-occurring human GLP-1 highly unfeasible, several long-acting GLP-1R agonists have been developed in the recent years to treat T2D, with the advantage of *1)* mimicking the endogenous GLP-1 effects and *2*) being more resistant to DPP-IV-mediated degradation [155]. In fact, mice with targeted downregulation of DPP-IV were described to have increased plasma GIP and GLP-1 levels, as well as insulin exocytosis, which were accompanied by a lower glucose release[156]. Importantly herein, several clinical studies described that exenatide or liraglutide (two GLP-1R agonists currently used in T2D treatment) were more efficient in decreasing blood glucose levels in hyperglycemic T2D patients than sitagliptin (a DPP-IV inhibitor) [1,157], with the additional advantage of minimizing the risk of hypoglycemia and weight gain (in comparison with insulin and other oral anti-T2D drugs)[1]. Moreover, GLP-1R agonists appeared to restore β-cell function, a highly relevant issue, as frequently (by the time of T2D diagnosis) β-cell mass and function are already impaired[1]. However, these compounds are not free from some secondary effects, with some patients treated with exenatide reporting nausea, diarrhea [146,158] and, although controversial, pancreatitis [159].

Regarding DPP-IV inhibitors, clinical studies described their good tolerability, the advantages on their oral intake (instead of peripheral injection, as GLP-1R agonists) [131] and, despite their neutrality on body weight and gastric emptying, they lower fasting and postprandial glucose [160], as well as HbA_{1C} levels in T2D patients that simultaneously undergo metformin administration [161]. Besides incretins, other DPP-IV substrates include other gastrointestinal hormones, neuropeptides, cytokines and chemokines [146]. Amongst them, the neuropeptide Y (NPY) is widely known to affect lipid metabolism and adipogenesis and blockade of its receptor was shown to blunt the DPP-IV-mediated stimulation of lipid accumulation [139]. Amongst DPP-IV inhibitors, sitagliptin, saxagliptin and linagliptin have been approved by the Food and Drug Administration (FDA) for treatment of T2D patients, whereas vildagliptin is also available in the European Union and alogliptin in Japan [132]. At their recommended doses, a daily administration of these DPP-IV inhibitors was shown to blunt DPP-IV activity for at least 24 h (except vildagliptin, which required a twice daily administration) [162]. Additionally, DPP-IV inhibitors can be used either as monotherapy or together with other oral agents or insulin and they induced low overall hypoglycemia rates (comparable to those of GLP-1R agonists)[132]. Importantly, as GLP-1 analogues, DPP-IV inhibitors also become less effective as insulin resistance progresses and pancreatic β-cells function deteriorates [155].

1.2.4. GLP-1 mimetics and GLP-1R agonists as potentially efficient drugs against neurodegeneration and AD

It is widely known that GLP-1 is also ubiquitously expressed in CNS, especially in cortex, hippocampus, hypothalamus, striatum, substantia nigra, brain stem and subventricular zone (an area of adult brain neurogenesis) [155]. Therefore, it is not surprising that not only the main peripheral roles of GLP-1 might be centrally controlled, but also that this incretin may play a pivotal role in CNS. Moreover, as brain GLP-1 has been increasingly faced as a neuroprotective molecule, its receptors at CNS may represent promising targets against neurodegeneration/death and learning/memory disturbances, *ie*, a putative pharmacological strategy against age- and/or T2D-related neurodegenerative diseases may necessarily involve GLP-1R. Accordingly, During *et al.* (2003) [163] reported that knockout mice for GLP-1R had memory and learning deficits.

1.2.4.1. GLP-1 mimetics: liraglutide

Amongst the already marketed GLP-1 analogs, liraglutide is the main long-acting compound, being used either as a monotherapy or in combination with metformin or thiazolidinediones (TZDs)[132]. Structurally, liraglutide shares 96% similarity with human GLP-1 and, when combined with sulphonylureas (SUs), decreases the risk for hypoglycemia [132]. Importantly, liraglutide-induced prevention in hippocampal synaptic loss was accompanied by restoration of synaptic plasticity and memory function in the APP/PS1 mouse model of AD [164]. Additionally, liraglutide decreased the formation of Aβ plaques[165] and the inflammatory response (by activating less astrocytes and microglia) [166], and promoted neurogenesis/neuronal proliferation in dentate gyrus [167]. Although these results point towards the beneficial use of liraglutide as an anti-AD pharmacological approach, further clarifying research is needed, with a particular emphasis on the ongoing clinical trials (NCT01469351 and NCT01843075, according to www.clinicaltrials.gov).

Given all the interesting features of incretin hormone's analogues (particularly their uniqueness of promoting a glucose-dependent insulin secretion, minimizing the damage associated with repeated hypoglycemia episodes), intense research efforts have been done in the recent years to develop and test other similar drugs, that may also represent some neuroprotective potential, as described elsewhere.

1.2.4.2. GLP-1R agonists: exendin-4, lixisenatide and albiglutide

Besides GLP-1 analogues (as liraglutide), the incretin class of anti-T2D drugs also include the GLP-1R agonists. Amongst the novel GLP-1R agonists, lixisenatide and albiglutide are currently undergoing phase III trials. Lixisenatide is based on exendin-4(1–39), with a modified C-terminal containing six additional lysine residues that not only increase its affinity to GLP-1R by 4-fold compared to the human GLP-1, but also extends its half-life to 3h [168,169]. Importantly, lixisenatide was already shown to cross the BBB into the CNS, thereby preventing Aβ-related impaired synaptic plasticity, hippocampal long-term potentiation (LTP) and spatial learning and memory deficits, most likely via the PI3K/Akt/GSK3β pathway [170]. Additionally, lixisenatide has been described to exert strong neurogenic effects in an AD rodent model [167,171].

Albiglutide consists of two GLP-1(7–36) molecules connected to recombinant human albumin[172], with a single amino acid substitution (ala→ gly) that renders it resistant to DPP-IV and with a half-life of ~5 days (which allows for a weekly dosing) [173]. Unfortunately, its size renders albiglutide unable to cross the BBB, also affecting its potential in body weight reduction and gastrointestinal tolerability[174]. Its secondary effects include nausea and injection site reactions [132].

Importantly herein, one of the most widely clinically used and best studied incretin-based therapy is exenatide (exendin-4, Ex-4), whose high efficiency against T2D and CNS damage have been increasingly demonstrated [175]. Exenatide is a synthetic, injectable GLP-1R agonist derived from Ex-4, a peptide obtained from the saliva of the Gila monster (*Heloderma suspectum*), being also resistant to DPP-IV [176]. Although Ex-4 only shares only a 53% amino acid sequence homology with human GLP-1, its effectiveness in lowering glucose levels is by far higher [146]. Therapeutically, Ex-4 may constitute an important supplement to diet and exercise in improving glycemic control in T2D adults [1], either as a monotherapy or in combination with other oral agents [177]. Ex-4 also reduces HbA_{1C} levels by 0.8–0.9%, decreases food intake [178] and, together with metformin and/or SUs, was shown to reduce body weight[179]. Strikingly, exenatide was also shown to promote graft survival and function in newly islet-cell transplanted T1D patients [180]. Although the underlying mechanisms remain poorly understood, it is plausible that, similarly to endogenous GLP-1, Ex-4 binds to (and activates) GLP-1R on pancreatic β-cells, thereby activating adenyl cyclase and rapidly increasing cAMP levels, with the subsequent stimulation of intracellular signaling cascades [111] that may culminate in the modulation of pancreatic β-cells' proliferation and function, together with an inhibition of apoptosis [146,147,178]. It is plausible that these actions of Ex-4 may occur together with its potent effects on glucose-dependent insulin secretion and insulin gene expression [181]. Nevertheless, some transient nausea and occasional vomiting have been frequently reported as Ex-4 side effects[182].

Regarding its effects on CNS, it has been shown that Ex-4's stability in blood (being insensitive to food deprivation for 24h[183]) and high lipophilicity allowed most of the peripherally-injected Ex-4 to cross the BBB without being trapped by its endothelial cells, thus rapidly reaching the brain intact [147]. Besides this, the

ubiquitous GLP-1R expression in CNS [184] further suggests that its activation and the downstream signaling cascades may at least partially be involved in Ex-4-mediated stimulation of neurogenesis in subventricular zone [185], neurite outgrowth, neuronal differentiation, rescue of degenerating neuronal cells and protection against both *in vitro* and *in vivo* excitotoxic damage [186]. Ex-4 also improved hippocampus-associated behavior in adult rodents [187] and protected against neuroinflammation [158]. More strikingly, Ex-4 was described to protect against Aβ-associated hippocampal neuronal death and rescue learning and memory in intracerebroventricularly-injected streptozotocin rats (a model of sporadic AD) [1]. In this perspective, besides its already proven important clinical impact against T2D, Ex-4 (or even other incretins) could also constitute a promising therapeutic strategy against AD, with the some expectation existing on the results from the ongoing clinical trial (NCT01255163, according to www.clinicaltrials.gov).

2. HYPOTHESIS AND AIMS

From the above, we hypothesized that Ex-4 restores the T2D-associated alterations in intracellular quality control mechanisms, neurodegeneration and death, thereby improving synaptic function and, ultimately, cognitive function.

Therefore, our main goal was to analyze the potential neuroprotective effect of chronic, continuous peripheral Ex-4 administration in middle-aged T2D rat brain. In this perspective, we analyzed the effect of chronic peripheral Ex-4 treatment in T2D: *1)* pathological features (glycemia, rate of glucose clearance from blood, plasma insulin levels and insulin resistance, HbA_{1C} levels); *2)* other T2D-associated risk factors (as body weight, hypercholesterolemia, hypertriglyceridemia and high blood pressure), as well as in *3)* rat brain cortical markers of oxidative stress, *4)* synaptic (dys)function, *5)* cellular quality control (autophagic) mechanisms and *6)* neurodegeneration and caspase-dependent and –independent apoptotic and necrotic cellular death.

Since long ago, rodent models have been the preferred and most widely used animal models of human pathologies, being regularly used to study neurological pathogenic mechanisms (due to the several similarities with human brains) and the overall effects of experimental therapies aiming at preventing or curing such diseases. Herewith, we used middle-aged (8 month old) non-diabetic male Wistar and T2D Goto-Kakizaki (GK) rat brain cortical homogenates, either submitted or not to the continuous peripheral delivery of the GLP-1R agonist Ex-4, for 28 days. GK rats are one of the most well known rodent models of T2D [188], resulting from the selective breeding of Wistar rats with oral glucose intolerance [189]. GK rats develop oral glucose intolerance as early as 2 weeks of age, exhibiting elevated plasma glucose levels upon administration of a glucose load at 4 weeks of age. By the 12 weeks of age, GK rats exhibited a T2D profile, characterized by elevated fasting glucose and insulin levels and a prolonged elevation in plasma glucose levels after an oral glucose load [188]. Since these animals are non-obese and spontaneously T2D, displaying also a defective insulin action and long-term complications similarly to those occurring in T2D humans [190], we strongly believe that they may constitute a highly valuable model to analyze the chronic effects of T2D *per se* on CNS, without the interference of other risk factors (*e.g*. obesity, hypertension).

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Micro osmotic Alzet® 2ML4 pumps were from Durect Corporation (Cupertino, CA, USA). Commercial glucometer (Glucometer-Elite) and compatible reactive tests (Ascencia Elite), as well as the A_{1C} Now HbA_{1C} Multi-Test were purchased to Bayer (Portugal). Rat Insulin EIA Kit was from SPI-BIO, Bertin Pharma (Montigny le Bretonneux, France). LE 5001 Non Invasive Blood Pressure meter was obtained from Panlab, Harvard Apparatus (Holliston, Massachusetts, USA). Accutrend kit for cholesterol and triglycerides and compatible stripes, PhosSTOP Phosphatase Inhibitor Cocktail Tablets and Complete, Mini, EDTA-free Protease Inhibitor Cocktail Tablets in easy packs were from Roche Diagnostics (Portugal). Rat GLP-1 ELISA kit was bought to Elabscience Biotechnology Co.,Ltd (Wuhan, China). QuantiChrom Glucose Assay Kit was from BioAssay Systems (Hayward, CA, USA). Polyvinylidene difluoride (PVDF) membranes (Immobilon®-P PVDF transfer membranes 0,45 μm) were purchased to Merck-Millipore (Germany). protease from *Bacillus licheniformis* (Type VIII), rabbit polyclonal anti-LC3 antibody, mouse monoclonal anti-synaptophysin, mouse monoclonal anti-actin, caspases-1 (N-acetyl-Tyr-Val-Ala-Asp-P-nitroanilide, Ac-YVAD-pNA) and -2 (N-acetyl-Val-Asp-Ala-Val-P-nitroanilide, Ac-VDAV-pNA) were from Sigma (USA). Ex-4 (Exenatide), mouse monoclonal anti-beclin 1, rabbit polyclonal anti-RIP1, rabbit polyclonal anti-RIP3 and rabbit polyclonal anti-GLP-1R were all from Abcam (Cambridge, UK). Rabbit polyclonal anti-P-mTOR antibody, mouse monoclonal anti-mTOR, rabbit monoclonal anti-PI3K class III, rabbit polyclonal anti-BAX and anti-Bcl2, rabbit polyclonal anti-caspase 12, rabbit monoclonal anti-PSD-95 and rabbit monoclonal anti-α-tubulin were purchased to Cell Signaling (The Netherlands). Mouse monoclonal anti-cytochrome c was from BD Pharmingen (San Diego, CA, USA). Anti-rabbit and anti-mouse IgG secondary antibodies and ECF fluorescence reagent were from GE Healthcare (Piscataway, NJ, USA). Protein A PLUS-Agarose Immunoprecipitation Reagent and rabbit polyclonal anti-TOM20 was from Santa Cruz Biotechnology (Dallas, Texas, USA). 8-Hydroxy-2-deoxy Guanosine EIA kit was obtained from Cayman Chemical (Pittsburgh, USA). Colorimetric substrates for caspases-3 (N-acetyl-Asp-Glu-Val-Asp-P-nitroanilide, Ac-DEVD-pNA), -8 (N-acetyl-Ile-Glu-Thr-Asp-P-nitroanilide, Ac-IETD-pNA) and -9 (N-acetyl-Leu-Glu-His-Asp-P-nitroanilide, Ac-LEHD-pNA) were purchased to Calbiochem (Darmstadt, Germany). All other reagents were of the highest grade of purity commercially available.

2.2. Animal characterization

Following Directive 2010/63/EU, Portuguese legislation and ethical approval provided by our institution's ethical committee, all efforts were made to reduce the number of animals used and to minimize animal suffering. Therefore, thirteen non-diabetic male Wistar rats (controls) of sixteen weeks old, obtained from Charles River (Barcelona, Spain), and twenty-six male, spontaneously T2D GK rats of sixteen weeks old, from Taconic Europe (Lille Skensved, Denmark) were housed in our Animal Research Center Laboratory (Faculty of Medicine/Center for Neuroscience and Cell Biology, University of Coimbra), under controlled light (12 h day/night cycle) and humidity conditions, and with free access to powdered rodent diet (Diet 4RF21 GLP, Mucedola Srl, Settimo Milanese, Italy) and water. After one month quarantine and adaptation period, animals' welfare, signs of distress and T2D parameters were periodically monitored, and glucose tolerance tests were used as selection index.

At 8 months old and before the treatment started, both Wistar and GK rats were physically characterized

(body weight) and relevant blood parameters (as glycemia, HbA_{1C}, blood pressure, glucose tolerance test (GTT), insulin levels and HOMA-IR) were determined after a 6-hour fasting period during the morning. After a 72h resting period, surgical implantation of micro osmotic pumps containing Ex-4 was performed, as described below.

2.3. Peripheral surgical implantation of Ex-4-containing micro osmotic pumps

All surgical procedures were performed under anesthesia with isofluorane atmosphere and local butorphanol injection. Briefly, thirteen Wistar rats and twenty-six GK rats were subcutaneously implanted with a micro osmotic pump, after a small incision in the skin between the scapulae [191], according to manufacturer's instructions. Rats were divided into three experimental groups. In one group (n=13), GK rats were continuously infused with Ex-4 (5 μg/kg/day; infusion rate 2.5 μl/h), for 28 days (from the 8^{th} to 9^{th} month of age)[192]. The remaining two groups (n=13 Wistar and n=13 GK rats) received saline infusion. Accuracy of micro osmotic pumps was verified according to manufacturer's instructions and also by weighing each pump before implantation and after removal from the animal.

Treatment success and pump implantation safety involved a regular monitorization of body weight, relevant blood parameters (as glycemia, HbA_{1C}, GTT, HOMA-IR, cholesterol, triglycerides), as well as blood "metabolic" hormones (*e.g.* insulin) (all these before and after treatment), thus ensuring that T2D hallmarks were not affected by pump implantation *per se* [191,192]. At 9 months old (and after post-treatment *in vivo* monitorization and behavioral analysis), rats were euthanized under sodium pentobarbital anesthesia before sacrifice by cervical dislocation and decapitation (EU guideline 86/609/EEC).

2.4. Body weight

Body weight was monitored once/week, in early afternoon, from 8 months (pre-treatment) to 9 months of age (post-treatment) and results were expressed as body weight (g).

2.5. Blood collection and plasma isolation

Blood was collected either from caudal vein (in living, anesthetized rats), once/week (before, during and immediately after treatment), or directly from the heart by transcardial punction (immediately after animal's sacrifice) with a heparinized syringe. Plasma was obtained by centrifugation at 14 000 rpm, for 2 min, at 4 °C, in a 2-16PK Heated and Refrigerated Centrifuge (Sigma, Germany), to determine insulin levels and HOMA-IR.

2.6. Blood glucose levels

Blood glucose levels were determined by a glucose oxidase reaction, using a commercial glucometer and compatible reactive tests, before, during (once/week) and after treatment (from caudal vein blood), as well as immediately after animals' sacrifice (intracardial punction blood). Results were expressed as mg glucose/dl blood.

2.7. GTT test

GTT analyses the rate of glucose clearance from blood [193]. Briefly, food was removed at about 8 a.m. (after nocturnal eating period) and rats were kept fasted for 6 h, being GTT test performed after 2 p.m. (resting period). Basal glycemia was measured before *D*-glucose injection, corresponding to time 0. Then, an intraperitoneal injection of 2 mg *D*-glucose/g body weight was given upon rat immobilization by the back of the neck and glycemia were determined after 15, 30, 60 and 120 min. At the end of the test, cages were supplied with wet food. Results were expressed as mg glucose/dl blood.

2.8. HbA_{1C} levels

Glycosylated hemoglobin (HbA_{1C}), an index of average glucose blood levels during the previous 2-3 months, was measured once/month (before and after treatment) in blood from caudal vein, using the kit A_{1C} Now Multi-Test with compatible reaction tests. Results were expressed as %.

2.9. Plasma insulin levels and insulin resistance

Plasma insulin levels were determined after treatment by using the Rat Insulin EIA Kit, according to manufacturer's instructions. Briefly, this kit is based on the competition between unlabelled rat insulin and acetylcholinesterase (AChE) linked to rat insulin (tracer) for limited specific guinea-pig anti-rat insulin antiserum sites, which then bind to the goat anti-guinea pig antibody attached to the well. The AChE tracer reacts with the Ellman's reagent forming a yellow compound, whose intensity is proportional to the amount of tracer bound to the well and inversely proportional to the content on free rat insulin. Thus, 100 µl of EIA buffer was added to Non Specific Wells (NSB) and 50 ul to Maximum Binding (Bo) wells. Then, 25 µl of each rat insulin standard and sample was added to the appropriate wells, followed by the addition of 25 µl of rat insulin AChE tracer (except to the blanks). Finally, 25 µl of rat insulin antiserum were dispensed to each well (except to blank and NSB wells) and incubated for 16-20 h, at 4ºC. After five washes with wash buffer, 200 µl of Ellman's Reagent were added and incubated in the dark for 180 min (corresponding to the time required for the Bo wells to reach 0.2-0.8 units of absorbance), at room temperature. Absorbance was read at 405 nm, in a Spectramax Plus 334 spectrophotometer (Molecular Devices, Silicon Valley, CA, USA) and insulin levels were calculated by plotting B/Bo (%) (y axis) *vs.* the concentrations of insulin standards (x axis). Results were expressed as ng insulin/ml plasma.

Insulin resistance degree was given by HOMA-IR calculation through the following formula:

HOMA-IR = (20*fasting insulin)/(fasting glucose-3, 5) and results were expressed as arbitrary units.

2.10. Blood cholesterol and triglycerides levels

Blood cholesterol and triglycerides levels were determined after and before treatment, after caudal vein or intracardial blood collection, using the respective Accutrend kit with compatible stripes. Results were expressed as mg cholesterol or triglycerides/dl blood.

2.11. Blood pressure

Blood pressure was measured using a LE 5001 Non Invasive Blood Pressure meter. The system used is based on the sphygmomanometric technique (the same used to take pressure in human beings). The unit features a pressure cuff, whose function is to occlude the passage of blood in the animal's tail, and a transducer which captures blood pulses. Generally, both are placed on the animal's tail to operate over the caudal artery. The LE 5001 detected the systolic or maximum pressure (SP) values, diastolic or minimum pressure (DP) values and the mean value, calculated as: MP=DP+0.33*(SP+DP). The heart rate was also picked up by the transducer. Its value was displayed continuously, and then stored along with the corresponding systolic, diastolic and mean pressure values. A measurement was comprised of the blood pulse (heart rate) and systolic and diastolic pressure values. Blood pressure was measured pre and post-treatment, before performing the glucose tolerance test.

2.12. Preparation of brain cortical membranes, cytosolic homogenates and isolated mitochondria

From the initial thirteen animals/group (Wistar placebo, GK placebo and GK plus Ex-4), brains from ten rats/group were immediately removed after animals' sacrifice, weighed and dissected into cortex, hippocampus (the most affected areas in AD [194]), hypothalamus and cerebellum. Cortices were immediately snap-frozen (-80ºC) for subsequent analysis (except for freshly-isolated brain cortical mitochondria, as described later). Then, a portion of cortex per brain hemisphere was slowly thawed, rapidly washed, minced and homogenized in lysis buffer containing (in mM): 25 HEPES, 2 $MgCl_2$, 1 EDTA, 1 EGTA (pH 7.5), supplemented with 100 μM phenylmethanesulfonylfluoride (PMSF), 2 mM dithiothreitol (DTT), PhosSTOP Phosphatase Inhibitor Cocktail Tablets and Complete, Mini, EDTA-free Protease Inhibitor Cocktail Tablets (the last ones according to manufacturer's instructions). Homogenization was done in a Potter-Elvejhem homogenizer with a Teflon pestle, at 300 rpm, 4ºC. Cortical homogenates were then centrifuged at 14 000 rpm for 10 min, 4ºC, in an Eppendorf 5415C centrifuge, to remove non-lysed tissue. The supernatants were then removed and pellets were centrifuged again at 14 000 rpm, for 10 min, 4ºC, to separate membrane and total cytosolic fractions. The resultant pellets (membrane fractions) and supernatants (cytosolic fractions) were further collected and assayed for protein content by the Sedmak method, as described below.

Regarding freshly-isolated brain mitochondrial fractions, we used a previously described procedure [195]. Briefly, a portion of the cortex was depleted from myelin and homogenized in a Potter-Elvejhem homogenizer with a Teflon pestle, in 10 ml of isolation medium containing (in mM): 225 mannitol, 75 sucrose, 5 HEPES, 1 EGTA (pH 7.4), supplemented with 1mg/ml fatty acid-free bovine serum albumin (BSA) and 750 μl protease from *Bacillus licheniformis*, Type VIII (E.C. 232-752-2), at 200 rpm, 4ºC. Then, an equal volume of isolation medium plus BSA fatty acid-free was added to the homogenate and centrifuged at 2500 rpm, 5 min, at 4ºC, in a Sorvall Evolution RC Superspeed centrifuge (Thermo Scientific, Waltham, Massachusetts, USA). The resulting pellet was discarded and supernatant was further centrifuged at 10 000 rpm, for 10 min, at 4ºC. Then, the resulting supernatant was discarded and the pellet was resuspended in 10 ml of isolation medium supplemented with BSA fatty acid-free and 50 μl of 20 mg/ml digitonin, and further centrifuged at 10 000 rpm, 10 min, at 4ºC. The supernatant was removed and the remaining pellet was carefully resuspended in 10 ml of isolation

medium supplemented with BSA fatty acid-free, being subsequently centrifuged at 10 000 rpm, 5 min, at 4ºC. Finally, 10 ml resuspension medium (containing, in mM: 225 mannitol, 75 sucrose, 5 HEPES (pH 7.4)) were added to the resulting pellet and, after a new centrifugation at 10 000 rpm, 5 min, at 4ºC, the new pellet was resuspended in 100 µl resuspension medium, collected, assayed for protein content by the Biuret method (as described below) and then kept at -80ºC for further analysis.

2.13. Protein quantification

To quantify protein there are several colorimetric or chromogenic methods available, whose utilization very commonly depend on protein composition as well as quantity. Regarding the protein composition, it can include amino acid content, any material bound covalently, such as carbohydrates, and protein conformation.

The Biuret method is based on the complex interaction of cupric ions with proteins. In this reaction, copper sulphate is added to a protein solution in strong alkaline solution and the complex formed between the cupric ions and the peptide bonds, has a purplish-violet color [196]. The reaction was named based on biuret and the similar colored complex it forms with cupric ions. To use this method, protein composition is not relevant because biuret reaction with proteins is independent on their composition. However, protein purity and association state could influence the results obtained with the biuret reagent [197].

After a 15 min incubation, the reaction extent was colorimetrically measured at 540 nm in a Spectramax Plus 334 spectrophotometer (Molecular Devices, Silicon Valley, CA, USA), and protein levels were determined by comparison with the standard curve. Results were given as mg protein/ml sample.

This technique was prepared accordingly:

Table I. Experimental procedure for protein quantification using the Biuret method

	BSA (0.4%) (µl)	**Sample (µl)**	**MilliQ H_2O (µl)**	**Ressuspension Buffer (µl)**	**Doc 10% (µl)**	**Biuret (ml)**
1	0	———	500	20	50	2
2	250	———	250	20	50	2
3	375	———	125	20	50	2
4	500	———	0	20	50	2
S1	———	20	500	———	50	2

The Sedmak method is another assay used to quantify proteins in solution and involves the use of low concentrations of perchloric acid ($HClO_4$) or other acids, allowing the proteins to remain soluble. This technique, first described by Sedmak and Grossberg [198] was prepared accordingly:

Table II. Experimental procedure for protein quantification using the Sedmak method

	MilliQ H_2O (µl)	**BSA 0.1% (µl)**	Protein (µg)	**Lysis Buffer supplemented (µl)**	**SEDMAK reagent (µl)**
P0	98	0	0	2	200
P2	96	2	2	2	200
P5	93	5	5	2	200
P10	88	10	10	2	200
P20	78	20	20	2	200
				Sample (µl)	
S1	98	———	———	2	200
S2	98	———	———	2	200

Sedmak reagent: 0,06% of Coomassie brilliant Blue G250 in 3% $HClO_4$

After a 15 min incubation, the reaction extent was colorimetrically measured at 620 nm in a Spectramax Plus 334 spectrophotometer (Molecular Devices, Silicon Valley, CA, USA) and protein levels were determined by comparison with the standard curve. Results were given as mg protein/ml sample.

2.14. Brain cortical GLP-1, insulin and glucose levels

Brain cortical levels of GLP-1 were determined using a sandwich-ELISA-based kit, according to manufacturer's instructions. Briefly, 20 µl of brain cortical homogenates (prepared as above-mentioned) or standards were pipetted onto a provided micro ELISA plate pre-coated a specific antibody against GLP-1, mixed and incubated for 90 min at 37ºC. Then, the liquid was removed and 20 µl of biotinylated detection antibody working solution was added to each well and incubated for 1 hour at 37ºC. After three washes with wash buffer solution, 20 µl Avidin-Horseradish Peroxidase (HRP) conjugate working solution were added and

incubated for 30 minutes at 37ºC. After five new washes, 90 µl substrate solution were added to each well and incubated for about 15 minutes at 37ºC, protected from light. During this step, wells containing GLP-1, biotinylated detection antibody and Avidin-HRP conjugate became blue colored. The enzyme-substrate reaction was stopped by addition of 50 µl sulphuric acid solution (Stop solution) and wells turned yellow. Absorbance was then spectrophotometrically measured at 450 nm, in a Spectramax Plus 334 spectrophotometer (Molecular Devices, Silicon Valley, California, USA). GLP-1 levels in our samples were obtained by comparison with a standard curve containing known amounts of GLP-1. This kit's sensitivity was 9.38 pg/ml, with a detection range from 15.63-1000 pg/ml, a coefficient of variation <10% and recognizing both natural and recombinant GLP-1, but without significant cross-reactivity or interference between rat GLP-1 and analogues. Results were expressed as pg GLP-1/mg protein.

Brain cortical insulin levels, were determined using the above-mentioned Rat Insulin Enzyme Immunoassay Kit (please, see Section *2.9.*). Results were expressed as ng insulin/mg protein.

Glucose levels were also determined in brain cortical homogenates through the QuantiChrom Glucose Assay Kit, according to manufacturer's instructions. Briefly, 2.5 µl of each brain homogenate was diluted in 250 µl reagent and heated in a heat block for 8 min. Then, the sample mix was cooled down in cold water bath for 4 min and 200 µl of such sample mix were transferred into a 96-well plate. Absorbance was measured in a Spectramax Plus 334 spectrophotometer, at 630 nm. Calculations were made by plotting the obtained absorbance values (upon subtraction of blank absorbance) against known standard glucose concentrations. Results were expressed as mg glucose/mg protein.

2.15. Western blotting analysis of brain cortical GLP-1R, Bcl2, Bax, cytochrome c, caspase-12 cleavage, LC3II, P-mTOR, beclin-1, PI3K III, synaptophysin, PSD-95, RIP3 and 1

Brain cortical total cytosolic homogenates or, whenever specified, brain cortical isolated mitochondria (50 µl), all prepared as previously described, were denatured with SDS sample buffer (containing 0.7 M 4x Tris.Cl/SDS pH 6.8, 30% glycerol, 10% SDS, 0.6 M DTT and 0.012% bromophenol blue), at 100ºC, for 5 min and then subjected to SDS/polyacrilamide gel (8, 10 or 15%, composed 1.5 M Tris (pH 8.8), 40% acrylamide, 10% (m/v) SDS, 10% ammonium persulfate (APS) and TEMED) electrophoresis (SDS-PAGE), at 130/140 V, in a buffer containing (in mM): 25 Tris-HCl (pH 8.0-8.5), 192 glycine and 0.1% (m/v) SDS (BioRad, Hercules, CA, USA). Then, samples were transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon®-P PVDF transfer membranes 0.45 µm), using a transfer buffer composed by 25 mM Tris-HCl (pH 8.0-8.5), 192 mM glycine, 20% (v/v) methanol and 0.005% (m/v) SDS (BioRad), for 90 min, at 0.75 A, 4ºC. The membranes were then blocked for 1 h at room temperature in 5% BSA (with 0.05% Tris-buffered saline (TBS)-Tween, which composition is 10% TBS 10x, 90% H_2O miliQ and 0.05% Tween-20). Membranes containing subcellular proteins were incubated overnight, at 4ºC, with rabbit polyclonal anti-LC3 antibody (1:1000, in 0.05% TBS-Tween plus 1% BSA), mouse monoclonal anti-beclin 1 (1:1000, in 0.05% TBS-Tween plus 1% BSA), rabbit polyclonal anti-P-mTOR antibodies (1:1000, in 0.05% TBS-Tween plus 5% BSA), rabbit monoclonal anti-PI3K III (1:1000, in 0,05% TBS-Tween plus 5% BSA), rabbit polyclonal anti-RIP1 (1:2000, in 0,05% TBS-Tween plus 1% BSA) and rabbit polyclonal anti-RIP3 (1:1000, in 0.05% TBS-Tween plus 1% BSA), rabbit polyclonal anti-BAX and anti-Bcl2 (1:1000, in 0.05% TBS-Tween plus 5% BSA), mouse monoclonal anti-cytochrome c (1:750, in 0.05% TBS-Tween plus 5% BSA), rabbit polyclonal anti-TOM20 (1:500, in 0,05% TBS-Tween plus

5% BSA), rabbit polyclonal anti-GLP-1R (1:1000, in 0.05% TBS-Tween plus 1% BSA), rabbit polyclonal anti-caspase 12 (1:1000, in 0,05% TBS-Tween plus 1% BSA), rabbit monoclonal anti-PSD-95 (1:1000, in 0.05 TBS-Tween plus 1% BSA), mouse monoclonal anti-synaptophysin (1:1000, in 0.05% TBS-Tween plus 1% BSA). Membranes were also labeled with mouse monoclonal anti-actin (1:5000, in 0.05% TBS-Tween plus 1% BSA) or rabbit monoclonal anti-α-tubulin (1:1000, in 0.05% TBS-Tween plus 5% BSA) antibodies, as loading controls. After the analysis of P-mTOR, membranes were further reprobed with mouse monoclonal anti-mTOR (1:1000, in 0.05% TBS-Tween plus 1% BSA). Then, membranes were washed three times with 0.05% TBS-Tween, for 5 min, and incubated with anti-rabbit or anti-mouse IgG secondary antibodies (1:10000 or 1:20000, in 0.05% TBS-Tween plus 1% BSA), for 2 h at room temperature, with gentle shaking, and washed again with 0.05% TBS-Tween for 5 min. Afterwards, membranes were developed using ECF fluorescence reagent. Immunoreactive bands were visualized by the Versa Doc Imaging System (BioRad, Hercules, CA, USA) and the fluorescence signal analysed using the QuantityOne software (BioRad, Hercules, CA, USA). Results were given as fluorescence intensity (INT)/mm^2, being then normalized to the respective loading control.

2.16. Co-immunoprecipitation of brain cortical IR and P-Tyr

Analysis of active IR in brain cortical homogenates was followed after immunoprecipitation of IR using Protein A PLUS-Agarose Immunoprecipitation Reagent, according to manufacturer's instructions with slight modifications. Briefly, cortical total cytosolic extracts (100 μg) were mixed (gentle shaking) with 40 μl of resuspended Protein A PLUS-Agarose and incubated for 30 min, at 4ºC. Then, beads were pelleted upon centrifugation at 2500 rpm in an Sorvall Evolution RC Superspeed centrifuge, for 5 min, at 4ºC, with 20 μl of the resulting supernatant (Total control) being collected and kept on ice until futher analysis. The remaining 20 μl of supernatant were then incubated with 5 μl rabbit monoclonal anti-IR (β-subunit) primary antibody for 1 h, at 4ºC. An additional 20 μl volume of resuspended Protein A PLUS-Agarose was then added to the previous mix and incubated for further 2 h, at 4ºC. After a centrifugation at 2500 rpm for 5 min, 4ºC, the pellet was collected and washed four times in 1 ml phosphate saline buffer (PBS), containing (in mM): 137 NaCl, 10 Na_2HPO_4, 1.8 KH_2PO_4, 2.7 KCl (pH 7.3), with 20 μl of the supernatant resulting from the second wash (Non-immunoprecipitated control) being collected and kept on ice until futher analysis. After the final wash, the supernatant was discarded and the pellet resuspended in 40 μl of 1x electrophoresis buffer (please, see Section *2.15*.), boiled for 2-3 min and centrifuged at 14 000 rpm, for 5 min, at 4ºC, using Spin-X® centrifuge tubes with filter (0.45 μm cellulose acetate in 2.0 ml polypropylene tube) to separate the Protein A PLUS-Agarose beads. Samples containing immunoprecipitated denatured IRs were immediately subjected to SDS-PAGE (10%) analysis, as described in Section *2.15*.

2.17. Analysis of membrane lipids and DNA oxidation

The extent of lipid peroxidation was determined by measuring thiobarbituric acid reactive substances (TBARS), using the thiobarbituric acid (TBA) assay [199], with slight modifications. This colorimetric assay detects, at 530 nm, the formation of a pink complex resulting from the reaction, in acidic medium, between TBA and malondialdehyde (MDA), a product of lipid oxidation.

Briefly, 10 μl of brain cortical homogenates were boiled in 100 μl of a peroxidation reagent containing

0.375% TBA, 15% tricholoacetic acid (TCA), 0,05% HCl 5M, for 10 min. Then, samples were cold on ice and centrifuged for 10 min at 3000 rpm, in an Eppendorf 5415C centrifuge. The resulting supernatant was collected onto a 96 well plate and the absorbance was measured at 530 nm in a Spectramax Plus 334 spectrophotometer, against a blank prepared in similar conditions, but in the absence of protein. The amount of TBARS formed was calculated using a molar extinction coefficient of $1.56x10^5$ $M^{-1}cm^{-1}$ and expressed as pmol TBARS/mg protein.

The levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG, a well known DNA oxidation marker) were determined by using the 8-Hydroxy-2-deoxy Guanosine EIA kit according to manufacturer's instructions. This immunoassay is based on the competition between 8-OHdG and a 8-OHdG-acetylcholinesterase (AChE) conjugate (8-OHdG Tracer) for a limited amount of 8-OHdG Monoclonal antibody. As the tracer amount remains constant and 8-OHdG varies, the amount of tracer able to bind to the monoclonal antibody is inversely proportional to the concentration of 8-OHdG in the sample. The complex antibody-8-OHdG binds to goat polyclonal anti-mouse IgG previously attached to the well. After washing to remove unbound reagents, Ellman's reagent (containing the substrate for AChE) is added to the well, yielding a yellow product that strongly absorbs at 412 nm and whose color intensity, spectrophotometrically measured, is proportional to the amount of 8-OHdG Tracer bound to the well, which is in turn inversely proportional to the amount of free 8-OHdG present in the well. Importantly, the 8-OHdG antibody used herein recognizes both free 8-OHdG and DNA-incorporated 8-OHdG.

Briefly, 100 μl and 50 μl EIA buffer were added to NSB and B0 wells, respectively. Then, 50 μl of brain cortical homogenates or 8-OHdG EIA standards were pipetted onto the respective wells, followed by the addition of 50 μl 8-OHdG AChE Tracer (except to Total Activity (TA) and Blank (Bl) wells) and by 50 μl 8-OHdG EIA Monoclonal Antibody (except to TA, NSB and Bl wells). After 18 h of incubation at 4ºC, wells were washed for five times with wash buffer and incubated with 200 μl Ellman's reagent (plus 5 μl tracer, only in TA wells), for 90 to 120 min (until B0 wells become 0.3-1.0 a.u., after Bl subtraction), with gentle shaking and protected from light. Absorbance was measured at 405 nm, on a Spectramax Plus 334 spectrophotometer. Sample concentrations of 8-OHdG were calculated after plotting the logit B/B0 for standards *vs.* log standard concentrations, followed by linear regression fit. Detection limit: 80% B/B_0: ~30 pg/ml. Sensitivity: 50% B/B_0: ~100 pg/ml. Results were expressed as pg 8-OHdG/mg protein.

2.18. Colorimetric activation of caspases-like activities

Conversion of procaspases into active caspases is generally accepted as one of the most reliable indicators of apoptosis, particularly in the case of the effector caspase-3 [200]. Caspases-3-, -8-, -1-, -2- and -9-like activities were determined by following a previously described procedure [201], with slight modifications. Briefly, twenty five (for caspases-3- and -8-like), forty (for caspases -1- and -2-like) or sixty-five (for caspase-9-like) μg of brain cortical cytosolic homogenates were added to a reaction buffer containing 25 mM HEPES, 10% (m/v) sucrose, 0.1% (m/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate (CHAPS) (pH 7.5), supplemented with 10 mM DTT and the respective colorimetric substrate (100 μM) for caspases-3 (N-acetyl-Asp-Glu-Val-Asp-P-nitroanilide, Ac-DEVD-pNA), -8 (N-acetyl-Ile-Glu-Thr-Asp-P-nitroanilide, Ac-IETD-pNA), -1 (N-acetyl-Tyr-Val-Ala-Asp-P-nitroanilide, Ac-YVAD-pNA), -2 (N-acetyl-Val-Asp-Ala-Val-P-nitroanilide, Ac-VDAV-pNA) and -9 (N-acetyl-Leu-Glu-His-Asp-P-nitroanilide, Ac-LEHD-pNA)-like activities. The reaction mixtures were incubated at 37ºC for 2 h, and the formation of pNA was measured at 405

nm in a Spectramax Plus 334 spectrophotometer. Caspases-like activities were expressed as percentage of control (caspase 1: 100% ± 19.95; caspase 2: 100% ± 37.54; caspase 8: 100% ± 22.64; caspase 9: 100% ± 10.94; caspase 12: 100% ± 12.26; 100% ± 8.703).

2.19. Data analysis and statistics

Data were analyzed and results were presented as mean ± SEM of the indicated number of independent experiments, each corresponding to one animal. Statistical significance was analyzed using the paired Student *t* test for single comparisons or non-parametric one-way ANOVA for multiple comparisons, with the Bonferroni post-test. $P < 0.05$ was considered significant.

CHAPTER 3

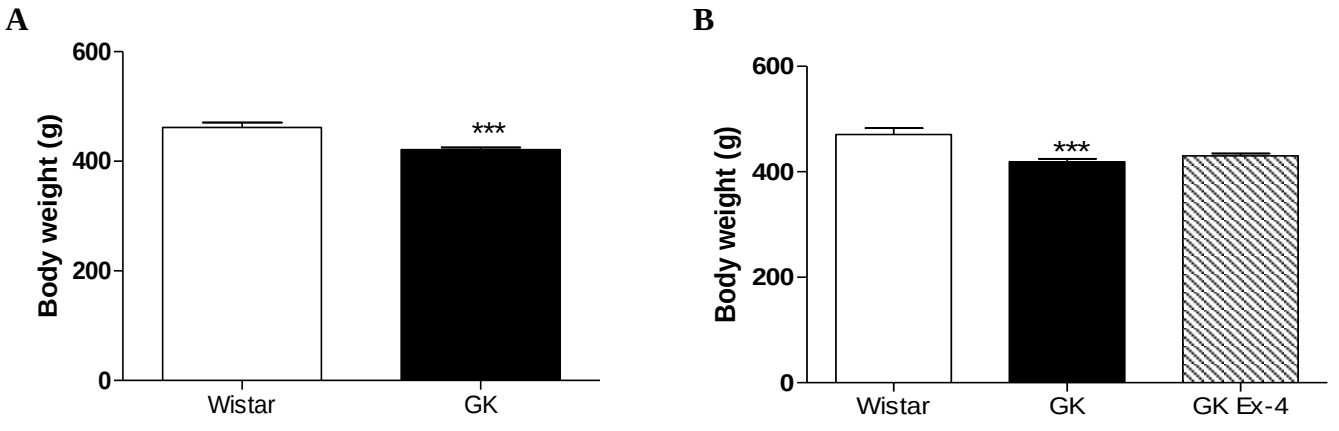
RESULTS [1]

[1] The results presented throughout this chapter were obtained in coauthoring with Emanuel Candeias MSc.

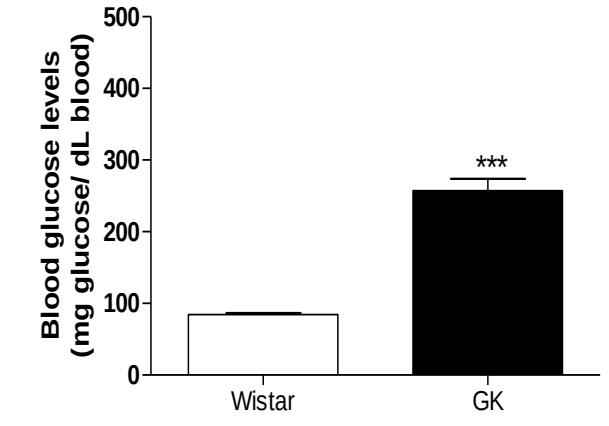
3.1. Animals' peripheral characterization

In this study, we used middle-aged (8 month-old), male, non-diabetic male Wistar (controls) and T2D GK rats. As described in *Materials and Methods* section, animals were monitored before and after treatment. We observed that, before treatment, GK rats had significantly lower body weight than Wistar ones (421.1± 3.88 *vs*. 461.9 ± 8.71 g in Wistar and GK rats, respectively) (Fig. 3.1.A). Although this difference was maintained after treatment, Ex-4 was not able to significantly restore body weight in GK rats to control values (Fig. 3.1.B). These results suggested that Ex-4 may not have an anorectic effect in our experimental conditions.

Regarding the evaluation of peripheral T2D hallmarks, occasional blood glucose, blood glucose clearance response after a glucose load (given by the GTT) and HbA_{1C} levels, pre-treated 8 month-old GK rats clearly showed a 3-fold higher glycemia (Fig. 3.1.C), a lower capacity to recover from a glucose load by clearing it from blood (given by their significantly higher blood glucose levels throughout the whole GTT test (Fig. 3.1.E) and their 3.4-fold higher area under the curve when compared to age-matched Wistar rats (Fig. 3.1.G)). This was also accompanied by an approximately 1.8-fold higher HbA_{1C} in GK rats before treatment (Fig. 3.1.I). Although by the end of treatment Ex-4-treated T2D rats had a 24% lower blood glucose levels than age-matched placebo GK rats, these differences did not reach statistical significance (Fig. 3.1.D). However, the recovery capacity of Ex-4-treated GK rats after the glucose load was highly improved after treatment, as given by the similarity in their blood glucose levels at the beginning and the end of the GTT test (Fig. 3.1.F) and by the 15% lower area under the curve in Ex-4-treated *vs.* placebo-treated GK rats (Fig. 3.1.H). Additionally, after a 28 days' treatment, Ex-4 also significantly decreased HbA_{1c} levels in T2D rats (from 8.29±0.19 to 7.34±0.28 % in placebo-treated *vs.* Ex-4-treated GK rats, respectively) (Fig. 3.1.E). Thus, these results suggested that chronic peripheral administration of Ex-4 was able to counteract one of the main T2D peripheral hallmarks: the hyperglycemic, glucose-intolerant profile of middle-aged GK rats.



C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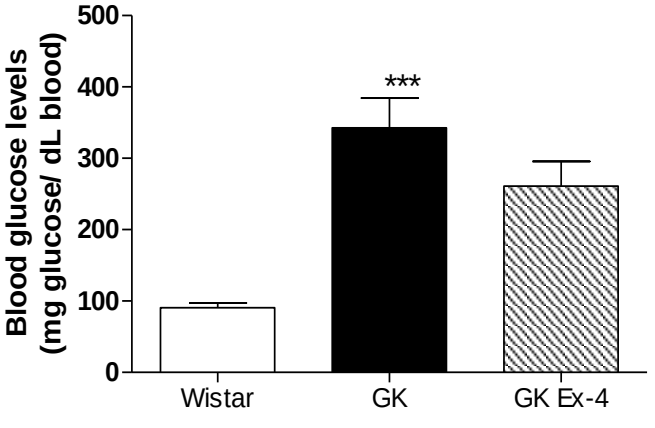
(mg glucose/ dL blood)
500
400
300
200
100
0
***
Wistar
GK

D


Blood glucose levels
(mg glucose/ dL blood)
500
400
300
200
100
0
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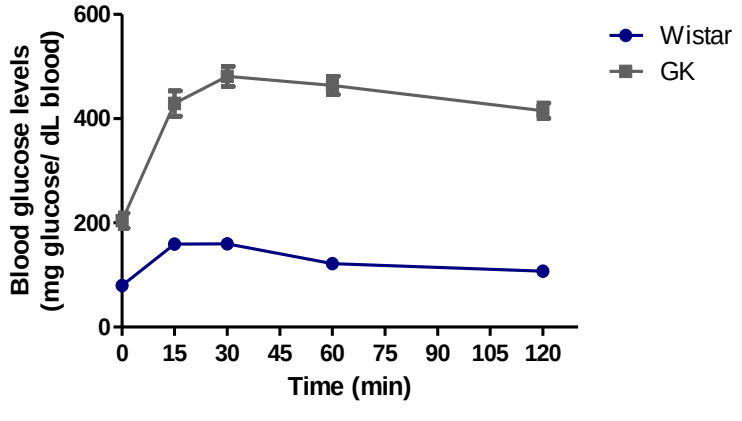
Wistar
GK
GK Ex-4

E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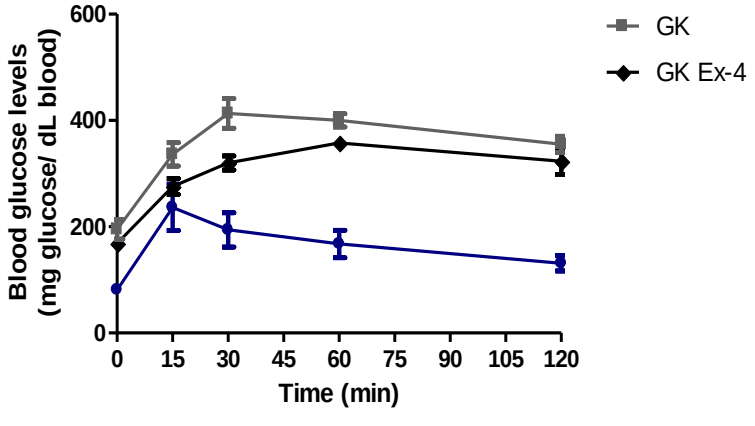
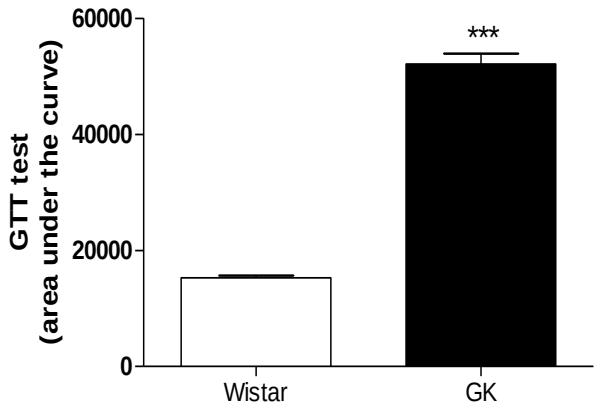
(mg glucose/ dL blood)
600
400
200
0
0 15 30 45 60 75 90 105 120
Time (min)
Wistar
GK

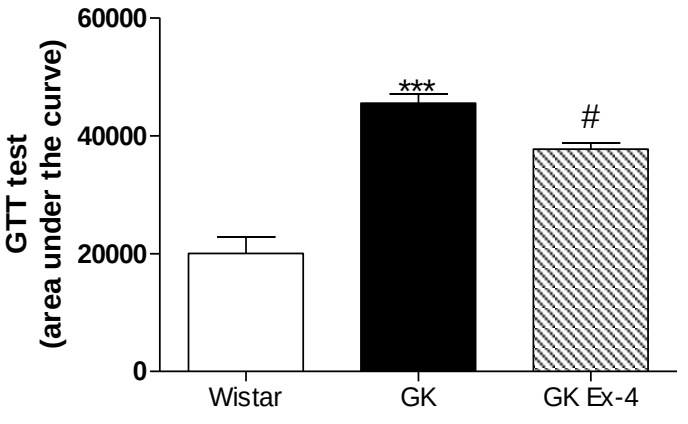
F


Blood glucose levels
(mg glucose/ dL blood)
600
400
200
0
0 15 30 45 60 75 90 105 120
Time (min)
Wistar
GK
GK Ex-4

G


GTT test
(area under the curve)
60000
40000
20000
0
***
Wistar
GK

H


GTT test
(area under the curve)
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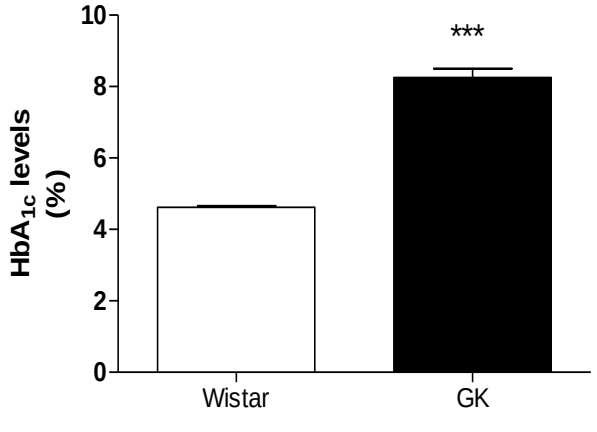
#
Wistar
GK
GK Ex-4

I


HbA1c levels
(%)
10
8
6
4
2
0
***
Wistar
GK

J

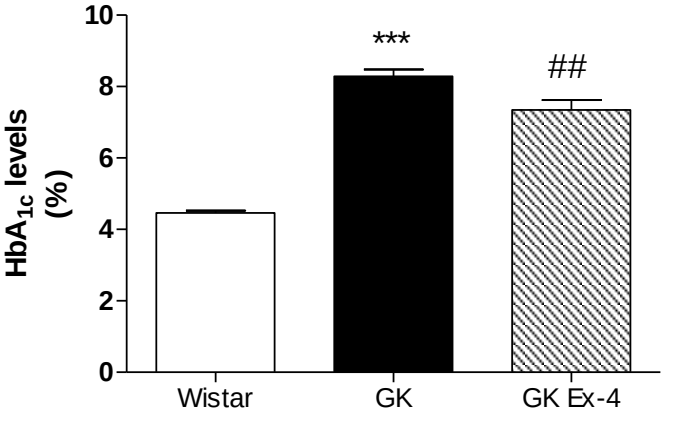

HbA1c levels
(%)
10
8
6
4
2
0
***
##
Wistar
GK
GK Ex-4

Figure 3.1. Animals' peripheral characterization. Body weight (A, B), occasional blood glucose levels (C, D), blood glucose clearance capacity (E-H) and glycosylated hemoglobin (HbA_{1c}) (I, J) were determined in non-diabetic Wistar and T2D GK rats before (A, C, E, G, I) and after treatment with Ex-4 (B, D, F, H, J). Middle-aged (8 month-old) Wistar and GK were subjected to the continuous subcutaneous delivery of 5 μg/kg/day Ex-4 (or placebo), for 28 days. Occasional blood glucose levels were obtained by the glucose oxidase reaction (from caudal vein blood), using a commercial glucometer and compatible reactive tests. Blood glucose clearance capacity was given by the GTT test, consisting on the measurement of caudal vein blood glucose levels before and 15, 30, 60 and 120 min after an intraperitoneal bolus of 2 mg *D*-glucose/g body weight, as well as on the determination of the overall GTT area under the curve. Glycosylated hemoglobin (HbA_{1C}) is an index of the average glycemias in the previous 2-3 months, being obtained from blood from caudal vein, using an appropriate kit and compatible reaction tests. Data are expressed as mean ± SEM of 9 to 26 animals per group. Statistical significance: ***P<0.001 *vs.* Wistar control rats; $^{\#}P$ < 0.05, $^{\#\#}P$<0.01 *vs.* placebo-treated GK rats.

Given that one of the primary effects of Ex-4 is to stimulate insulin secretion [181], we also evaluated its effect on GK rats' plasma insulin content and their insulin resistant profile (as given by the HOMA-IR profile) after treatment (Fig. 3.2.A and B). Regarding plasma insulin, we observed that its levels were approximately 1.6-fold higher in the placebo-treated T2D rats than in age-matched controls (in accordance with Moreira *et al.* (2007) [202], who described that, at the initial stages of the disease, GK rats may have a certain degree of hyperinsulinemia) and that chronic Ex-4 treatment was not able to further increase plasma insulin content in GK rats (Fig. 3.2.A). However, from the evaluation of insulin resistance, we observed that chronic Ex-4 induced a 32.9% decrement in HOMA-IR index in T2D rats compared to placebo-treated T2D rats (Fig. 3.2.B). Importantly, these results suggested that, as expected, our middle-aged GK rats were already insulin resistant (another feature of T2D) and peripheral Ex-4 therapy was able to ameliorate this parameter in GK rats.

Overall, these results suggested that the chronic peripheral continuous delivery of Ex-4 significantly ameliorated the main peripheral features of T2D in middle-aged GK rats.

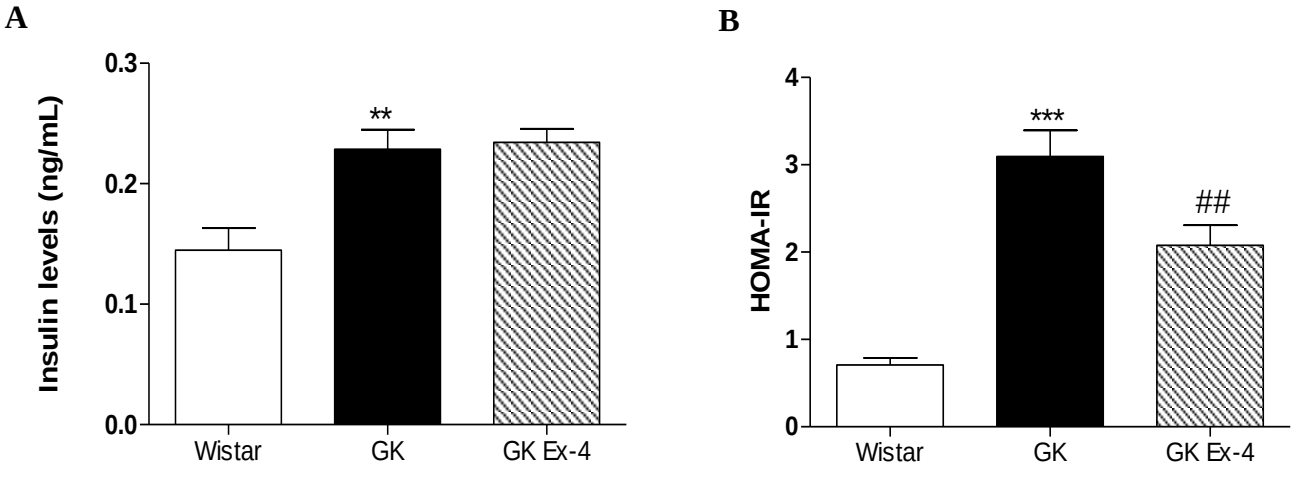


Figure 3.2. Effect of chronic continuous peripheral Ex-4 treatment on plasma insulin levels and insulin resistance in T2D GK rats. After treatment and animals' euthanasia, blood was collected by transcardial punction and plasma was obtained upon a 14 000 rpm centrifugation for 2 min, at 4ºC. Plasma insulin levels were determined by the Rat Insulin EIA Kit (according to manufacturer's instructions) and the HOMA-IR index calculated by using the formula: HOMA-IR = (20*fasting insulin)/(fasting glucose-3.5). Data are expressed as mean ± SEM of 11 to 13 animals per group. Statistical significance: **P<0.01, ***P<0.001 *vs.* Wistar control rats; $^{\#\#}P$<0.01 *vs.* placebo-treated GK rats.

As increased blood cholesterol and triglycerides are widely known to be increased upon T2D [180], we also evaluated the effect of Ex-4 on these parameters (Fig. 3.3. A-D). Although blood cholesterol levels were not significantly different between the experimental groups tested after treatment (Fig. 3.3. A), regarding blood triglycerides we observed that Ex-4 slightly (but not significantly) decreased triglyceride content in GK rat blood (Fig. 3.3.B). Regarding systolic blood pressure, as its values were highly variable between animals, we chose to establish different ranges of values and identify each range by an index, from 1 to 4. Thus, index 1 corresponded to blood pressure values minor than 90 mmHg (low blood pressure), index 2 ranged from 90 to 130 mmHg (normal blood pressure), index 3 ranged from higher than 130 to 300 mmHg (hypertension) and index 4 corresponded to blood pressure values higher than 300 mmHg (high blood pressure). We observed that systolic blood pressure degree was not different between the experimental groups in our procedure. Particularly, Ex-4 exerted no effect in blood pressure levels of the GK rats (Fig.3.3C). Overall, it seems only one animal of our population of GK rats was positively influenced by Ex-4 and had its values of blood pressure decreased. Therefore, these results suggest that, under our experimental conditions, Ex-4 may not significantly interfere with blood cholesterol and triglycerides' metabolisms. In addition, we also did not observe a significant effect of Ex-4 in GK rats' systolic blood pressures.

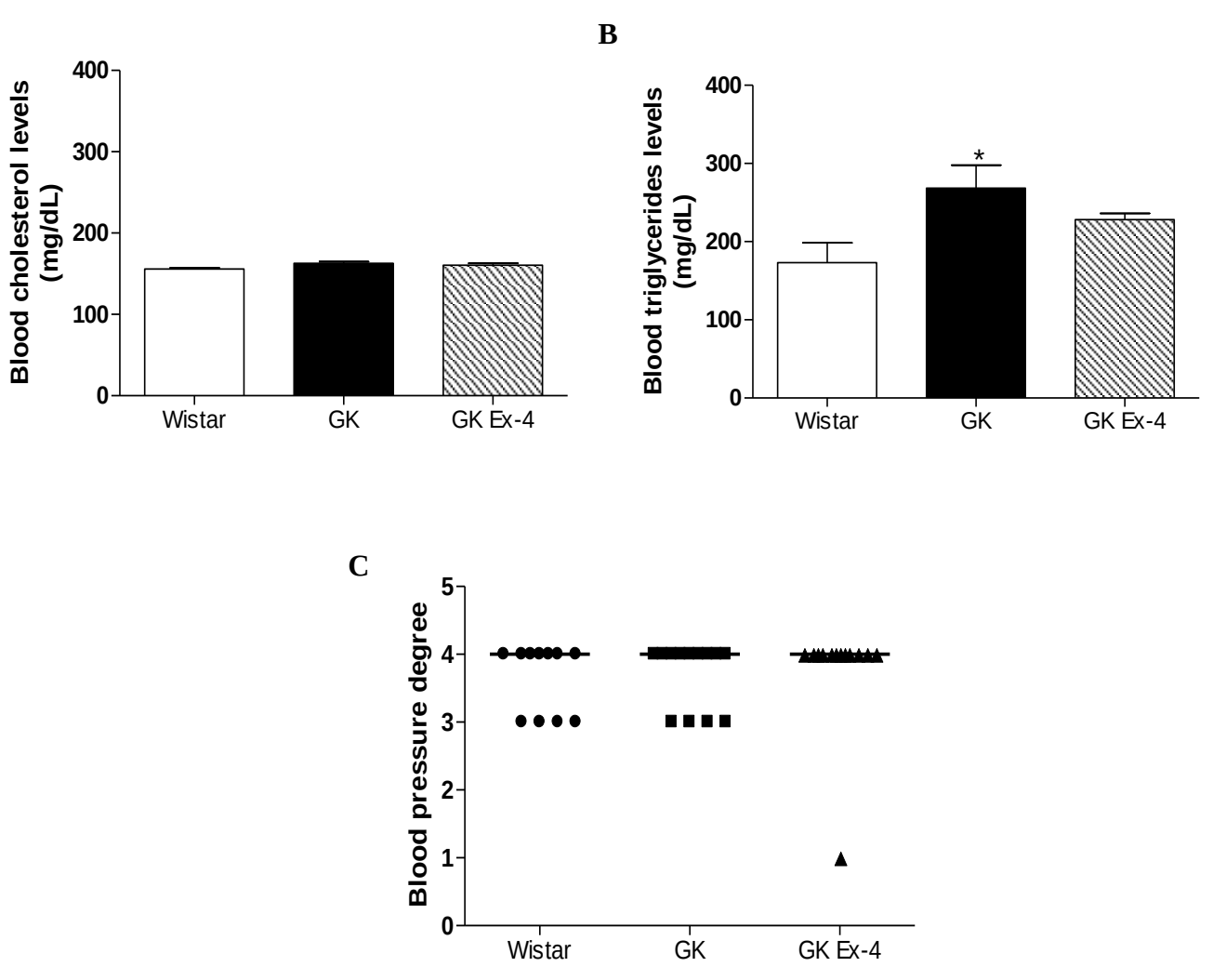


Figure 3.3. Effect of subcutaneous Ex-4 administration on blood cholesterol, triglycerides and pressure in T2D rats. Immediately after animals' euthanasia, blood was collected by transcardial punction and both cholesterol and triglycerides' levels were determined using the respective Accutrend kit with compatible stripes, as described in *Materials and Methods*. Systolic blood pressure was measured from rats' caudal artery, by a non-invasive sphyngomanometric technique composed by a tail pressure cuff and a transducer that captures blood pulses and sends the information towards a blood pressure meter. As systolic blood pressure values were highly variable between animals, we established scores

and identified each range of values in an index from 1 to 4. Thus, index 1 corresponded to blood pressure values lower than 90 mmHg (low blood pressure), index 2 ranged from 90 to 130 mmHg (normal blood pressure), index 3 ranged from higher than 130 to 300 mmHg (hypertension) and index 4 corresponded to values higher than 300 mmHg (high blood pressure). Data are expressed as mean ± SEM or median and respective quartiles (for systolic blood pressure scores) of 9 to 13 animals per group. Statistical significance: **P*<0.05 *vs.* Wistar control rats.

3.2. Chronic peripheral Ex-4 administration did not affect T2D GK rat brain weight

Immediately after the animals' sacrifice, their brain was collected and weighed, in order to assess if peripheral administration of Ex-4 affected brain mass. Although previous studies reported that T2D leads to a pronounced brain atrophy and, therefore, to a lower brain weight [2], we did not observe significant differences neither between placebo-treated control and GK rats nor between Ex-4-treated and placebo-treated GK animals (Fig. 3.4.). These results suggest that middle-aged GK rats did not suffer from macroscopically-detectable brain atrophy and, therefore, Ex-4 did not exert any significant effect herein.

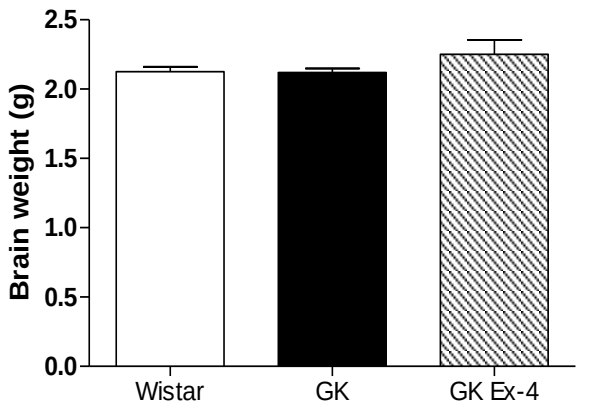


Figure 3.4. Effect of peripheral Ex-4 treatment on rat brain weight. Immediately after rats' euthanasia, brains were separated from the skull and weighed. Data are expressed as mean ± SEM of 8 to 10 rats per group.

3.3. Effect of chronic peripheral treatment with Ex-4 on GK rat brain cortical GLP-1 levels and GLP-1R protein expression

As Ex-4 is an agonist of GLP-1R, highly lipophylic and thereby able to readily cross the BBB and reach the brain intact, and GLP-1R is highly expressed in brain and activated by the drug [183], we measured both the levels of GLP-1 (Fig. 3.5.A) and the GLP-1R protein expression in brain cortical homogenates (Fig. 3.5.B). Although neither the 3.1-fold lower GLP-1 levels in placebo-treated nor their 2.4-fold increase in Ex-4-treated GK rat brain cortical homogenates reached statistical significance, these observations suggested that chronic subcutaneous delivery of Ex-4 for 28 days was sufficient to stimulate the synthesis of GLP-1 (either locally in brain and/or peripherally) (Fig. 3.5.A). However, these tendencies were not mirrored by the GLP-1R protein expression under the same conditions, as no significant differences were reported herein (Fig. 3.5.B).

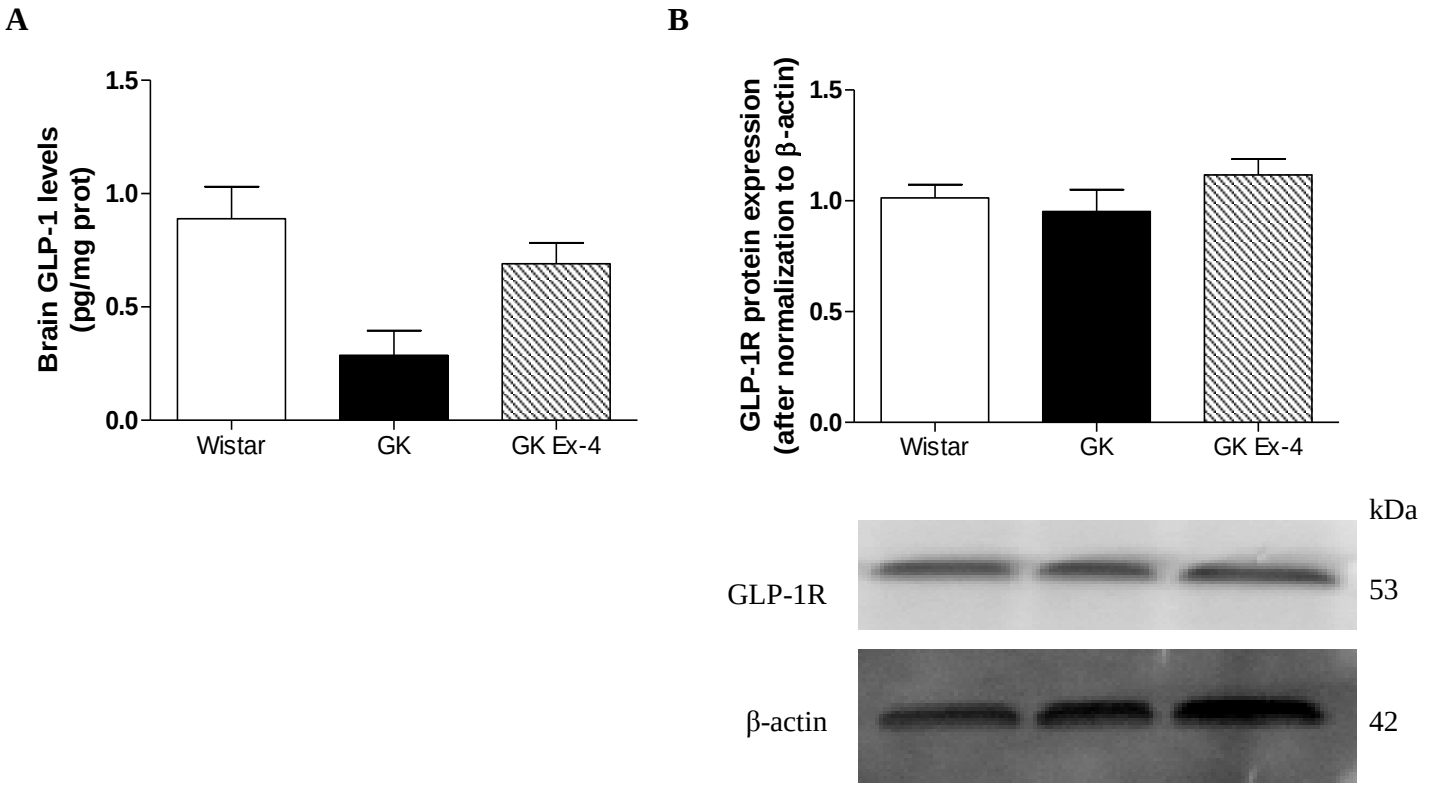


Figure 3.5. Effect of chronic subcutaneous Ex-4 administration on GK rat brain cortical levels of GLP-1 and GLP-1R protein expression. After animals' sacrifice, brain cortical homogenates were prepared as described in *Materials and Methods* and GLP-1 levels were measured by a sandwich-ELISA-based kit, according to manufacturer's instructions. GLP-1R protein expression was detected by SDS/PAGE (10%) electrophoresis followed by transfer into PVDF membranes and incubation with rabbit polyclonal anti-GLP-1R antibody. Then, membranes were reprobed with mouse monoclonal anti-β-actin. Data are expressed as mean ± SEM of six animals per group.

3.4. The role of chronic subcutaneous Ex-4 treatment on GK rat brain cortical insulin levels and IR protein expression

Given the widely described effect of T2D on insulin levels, that brain insulin resistance is a common molecular link between T2D and AD, and that Ex-4 is known by its peripheral insulinotropic effect [203], we also analyzed the effect of chronic peripheral Ex-4 administration on insulin content and its receptor activation in brain cortical homogenates from GK rats. Surprisingly, we observed that Ex-4 was not able to recover from the 44% decrease in GK rat brain cortical insulin levels (Fig. 3.6.A) and this was not accompanied by significant changes in IR activation (as given by its phosphorylation at tyrosine residues) (Fig. 3.6.B). These results suggest that the subsequent brain Ex-4 effects may not be mediated by a local increase in insulin levels nor by IR activation (at least in cortical region).

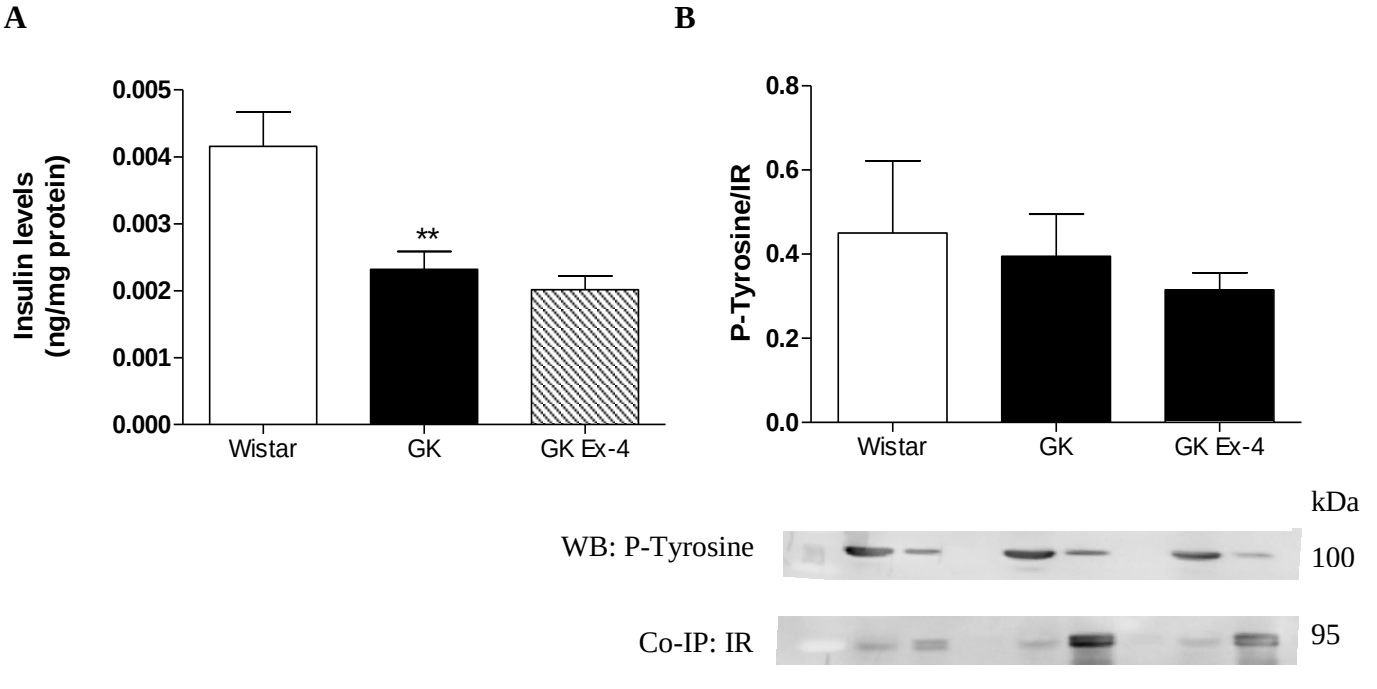


Figure 3.6. Effect of chronic subcutaneous Ex-4 therapy on T2D rat brain cortical levels of insulin and IR activation. After animals' sacrifice, brain cortical homogenates were prepared as described in *Materials and Methods* and insulin levels were measured by the Rat Insulin EIA Kit (according to manufacturer's instructions). IR activation was determined after co-immunoprecipitation of brain cortical homogenates with rabbit monoclonal anti-IR (β-subunit) antibody, followed by SDS/PAGE (10%) electrophoresis, transfer into PVDF membranes and ultimately incubation with mouse monoclonal anti-phospho-tyrosine antibody. Data are expressed as mean ± SEM of six or two animals per group, regarding insulin levels and IR activation, respectively. Statistical significance: $^{**}P<0.01$ *vs.* Wistar control rats.

3.5. Effect of chronic subcutaneous Ex-4 administration on GK rat brain cortical glucose levels

As hyperglycemia has been widely described to be one of the crucial players in T2D-related brain damage and chronic complications (including dementia and AD) [12,204] and given the anti-T2D effect of Ex-4 , we measured the brain cortical levels of glucose(Figure 3.7). Although placebo-treated GK rats had a 1.4-fold higher brain cortical glucose levels than age-matched placebo-treated Wistar rats, revealing that their brain glucose content was mirroring their peripheral hyperglycemia (Fig. 3.7.), and Ex-4 was able to restore GK rat brain glucose levels to values nearly those from control rats (0.012±0.001 mg glucose/mg protein), these differences did not reach statistical significance (Fig. 3.7.). Nevertheless, these results suggested that Ex-4 might be exerting an anti-hyperglycemic effect also in T2D CNS.

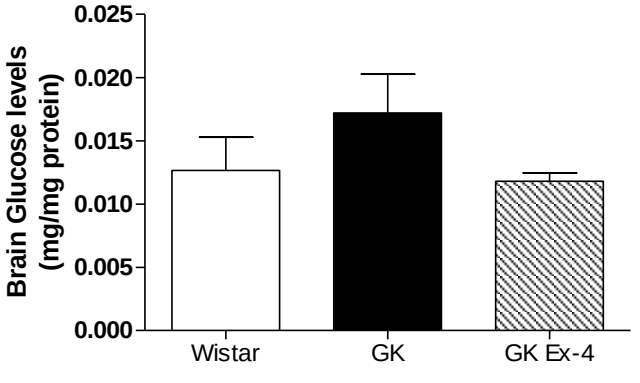


Figure 3.7. Effect of chronic subcutaneous Ex-4 administration on T2D rat brain cortical glucose levels. After animals' sacrifice, brain cortical homogenates were prepared as described in *Materials and Methods* and glucose levels were determined by the QuantiChrom Glucose Assay Kit, according to manufacturer's instructions. Data are expressed as mean ± SEM of six animals per group.

3.6. The role of chronic subcutaneous Ex-4 treatment on oxidative stress markers in GK rat brain cortex

Oxidative stress, resulting from an imbalance between the increased formation of ROS and/or impaired antioxidant defense mechanisms, has been widely described to play a deleterious role both in T2D and AD brain, leading to lipid, protein and DNA oxidation and subsequent cellular damage [11]. This is of the outmost importance, as brain is highly susceptible to oxidative injury [205]. As oxidative stress is a highly dynamic process, the successful detection of ROS levels/formation in tissues is often very difficult. Thus, we indirectly assessed oxidative stress in our experimental conditions by measuring the levels of other secondary byproducts, namely the TBARS and 8-OHdG, to evaluate both lipid and DNA oxidation, respectively (Fig. 3.8.A and B).

We observed that although TBARS levels were only 13.8% higher in placebo-treated T2D GK rat brain compared to placebo-treated Wistar rats, and Ex-4 lowered these values by 23%, these differences did not reach enough statistical significance (Fig. 3.8.A). Conversely, both placebo- and Ex-4-treated GK rats presented 17.5% and 18.9% lower 8-OHdG levels than placebo-treated Wistar and GK rats, respectively (Fig. 3.8.B). However, none of these differences reached statistical significance. These results suggested that Ex-4 was trying to counteract the T2D-related lipid and DNA oxidative injury in rat brain cortex.

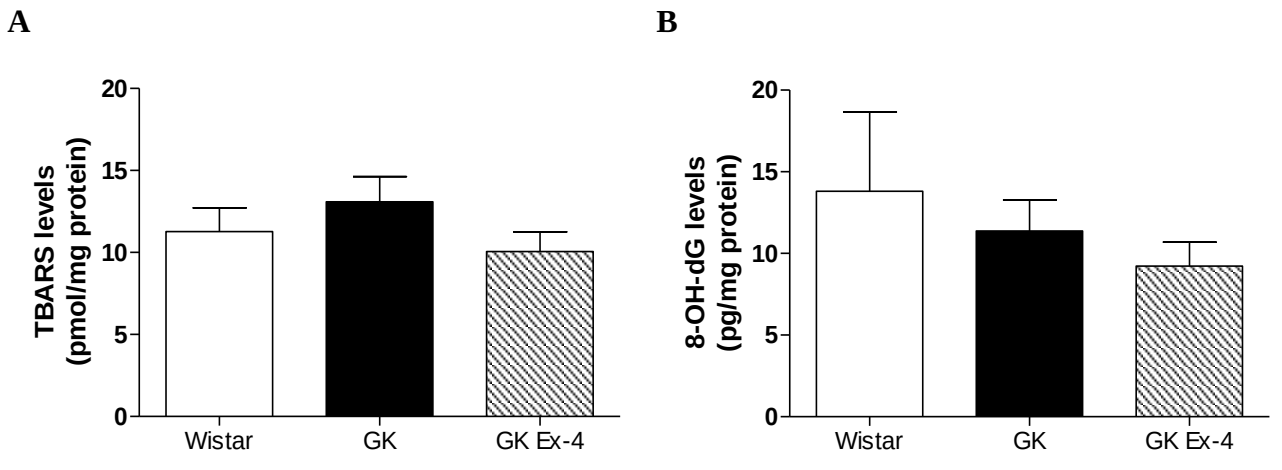


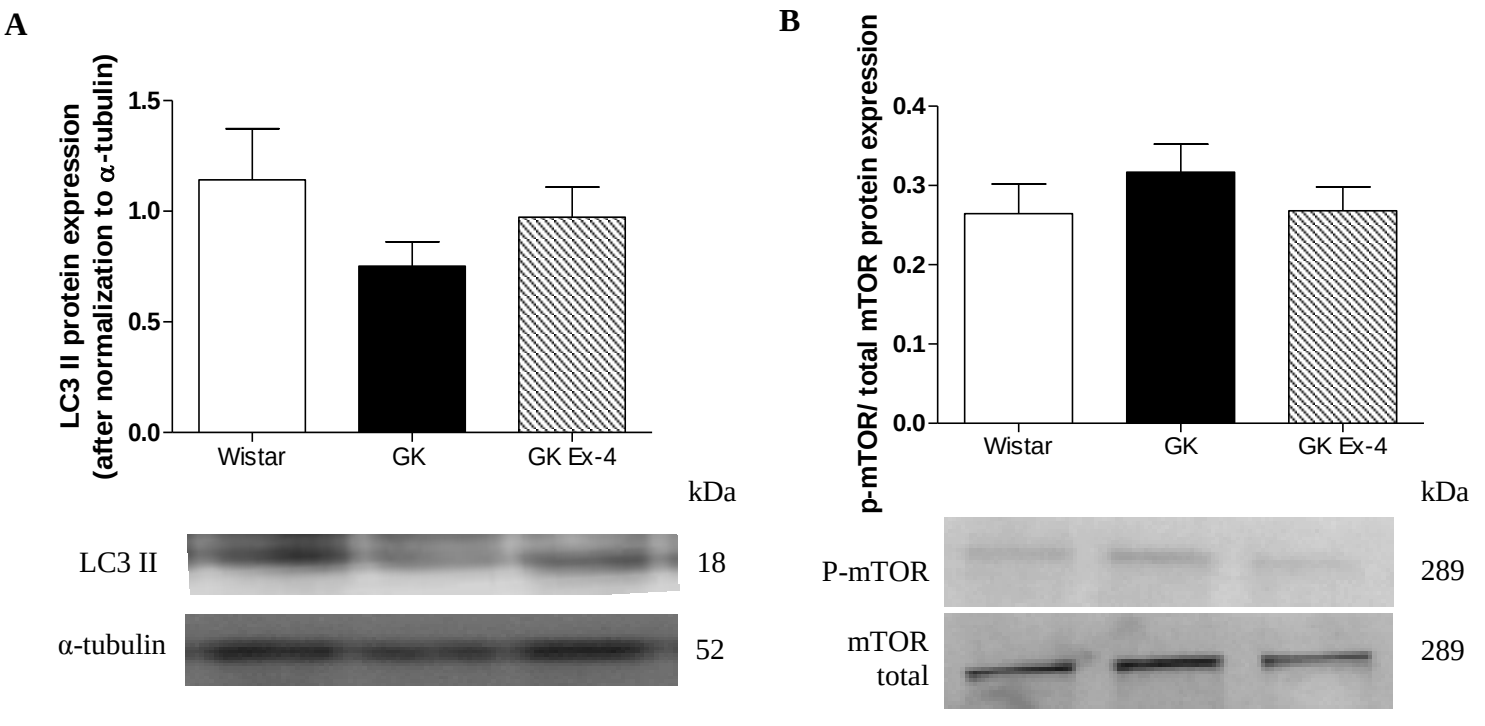
Figure 3.8. Effect of chronic subcutaneous Ex-4 administration on T2D rat brain cortical lipid and DNA oxidation. After animals' sacrifice, brain cortical homogenates were prepared as described in *Materials and Methods*. Lipid oxidation was given by the colorimetric detection, at 530 nm, of TBARS levels upon the formation of a pink complex resulting from the reaction, in acidic medium, between thiobarbituric acid and the lipid oxidation product malondialdehyde. DNA oxidation was given by the measurement of 8-OHdG by the 8-Hydroxy-2-deoxy Guanosine EIA kit according to manufacturer's instructions. Data are expressed as mean ± SEM of six animals per group.

3.7. Effect of chronic peripheral administration of Ex-4 on autophagic mechanism in GK rat brain cortex

Under physiological conditions, autophagy is a catabolic mechanism used for degradation of old and

damaged proteins and, therefore, contributing for cell survival; however, this process has been also recently implicated as a pivotal contributor for cell death upon severe damaging conditions [29]. As autophagy has been implicated in T2D or even AD pathologies, but the evidences remain controversial, we next evaluated if Ex-4 could have a (protective) effect on brain cortical autophagic mechanisms upon T2D.

First, we assessed protein expression levels of LC3-II in rat brain cortical homogenates (Fig. 3.9.A), one of the most commonly used markers of autophagy and a protein expressed in autophagosomes [48]. Although LC3-II protein expression was decreased in placebo-treated GK rat brains compared to age-matched placebo-treated Wistar rats (from 1.14±0.23 in control rats to 0.75±0.11 in T2D rats), and Ex-4 induced a 1.3- fold increase in LC3-II protein expression compared to placebo-treated GK rats (Fig. 3.9.A), these differences were not statistically significant. Then, we analyzed the protein expression levels of phospho-mTOR (P-mTOR), a key regulator of autophagy (Fig. 3.9.B). We observed that the placebo-treated T2D rat brains had 15.4% higher (although non-significant) P-mTOR/total mTOR than the placebo-treated controls, whereas Ex-4 returned its values to nearly control ones (Fig. 3.9.B). Another controller of the autophagic process is beclin-1 [49]. However, it is still unclear whether beclin-1 acts a negative or a positive regulator. Nevertheless, we determined its protein expression levels in rat brain cortical homogenates (Fig. 3.9.C). We observed that placebo-treated GK rats had a 25.6% lower beclin-1 expression than the placebo-treated Wistar rats, whereas in Ex-4-exposed GK rats its protein expression was only 9.3% higher than in non-treated GK rat brain (Fig. 3.9.C). However, none of these differences were statistically significant. Finally, another regulatory complex intrinsically involved in autophagy is the PI3K class III [49], whose protein expression was also evaluated in our experimental conditions (Fig. 3.9.D). Similarly to beclin-1, PI3K class III protein expression was lower in placebo-administered GK rat brains than in Wistar rats (from 1.08±0.29 to 1.63±0.29, in GK placebo *vs.* Wistar placebo, respectively), and Ex-4 treatment induced a slight increase in PI3K class III protein expression in GK rats (to 1.37±0.31) (Fig. 3.9.D). Although our results did not reach statistical significance, the tendencies observed suggested that the autophagic process could be slightly more activated in brain cortices from the Ex-4-treated GK rats, probably constituting a protective strategy against T2D-related brain degeneration and death.



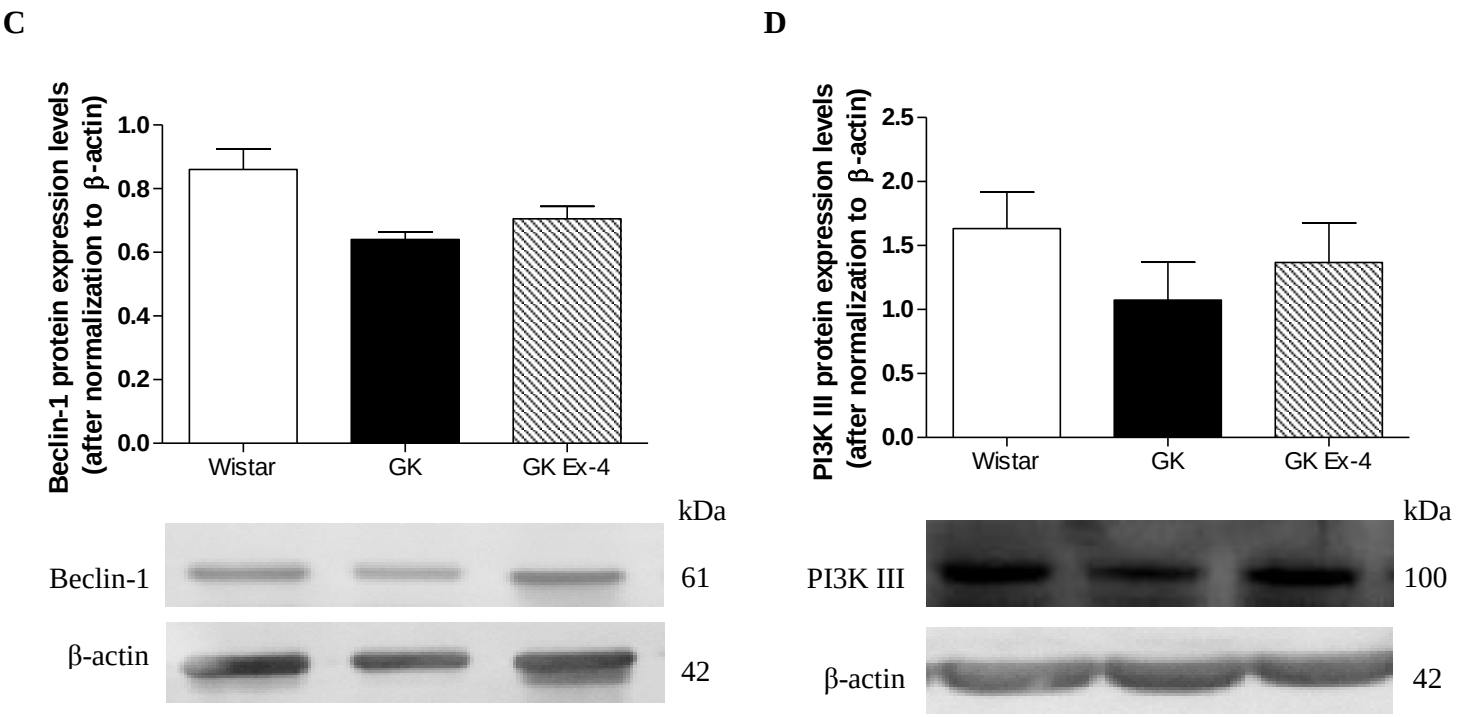


Figure 3.9. Effect of chronic subcutaneous Ex-4 administration on T2D rat brain cortical autophagic markers. After animals' sacrifice, brain cortical homogenates were prepared as described in *Materials and Methods*. LC3-II (A), P-Mtor (B), beclin-1 (C) and PI3K class III (D) protein expression were detected by SDS/PAGE (10% or 15%) electrophoresis followed by transfer into PVDF membranes and incubation with rabbit polyclonal anti-LC3, rabbit polyclonal anti-P-mTOR, mouse monoclonal anti-beclin 1 and PI3K class III antibodies. Then, membranes were reprobed with rabbit monoclonal anti-α-tubulin, mouse monoclonal anti-mTOR and mouse monoclonal anti-β-actin antibodies. Data are expressed as mean ± SEM of six animals per group.

3.8. Effect of peripheral exposure to Ex-4 on apoptotic cell death in GK rat brain cortex

Given our previous results on the possible Ex-4-induced slight activation of autophagy markers as an anti-cell death mechanism in T2D rat brain and some evidences on the anti-apoptotic role of Ex-4 [186,206–208], we next assessed its effect on caspase-mediated apoptotic activation (Fig. 3.10). From the analysis of the caspases-1- and -2-like activation (initiator caspases) [74], we observed that, despite the slight increase (32.9% for caspase-2-like activation) and the decrease (26.3% for caspases-1-like activation) in placebo-treated GK rats compared to placebo-treated Wistar rats, Ex-4 induced a 11.2% and 17.1% decrement in the activation of both caspases in GK rat brain, respectively (Fig. 3.10.A,B). However, none of these differences reached statistical significance. Regarding caspase-8-like, initiator caspase mostly involved in the extrinsic pathway of apoptotic cell death [209], we observed that T2D induced a significant increase in its activation (to 139.6±26.8% of control Wistar rats) (Fig. 3.10.C). However, Ex-4 administration was not able to counteract this effect in GK rat brain (Fig. 3.10.C). Another putative pathway to induce apoptotic cell death involves the endoplasmic reticulum-related caspase-12, whose role in AD has been increasingly described [91]. From the evaluation of caspase-12 cleavage, we did not observe significant changes between our experimental groups (Fig. 3.10.D).

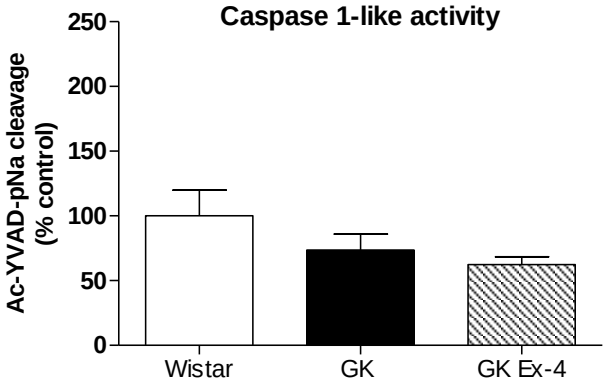
Besides the above-described initiator caspases, we also determined the activation of caspase-9-like, as this caspase is able to induce the downstream activation of the effector caspase-3 and, together with cytochrome c and the apoptotic protease-activating factor-1 (Apaf-1), caspase-9 forms the apoptosome complex [67].

Surprisingly, we did not observe significant changes between the different experimental conditions studied (Fig. 3.10.E).

Since the above results suggest that *1)* T2D may exert some pro-apoptotic effect in rat brain cortex, mostly via the activation of the caspase-8-like extrinsic pathway, but also in a minor degree through the slight activation of caspase-2-like, and *2)* Ex-4 slightly inhibited both caspases-1- and -2-like, we next evaluated the cleavage of the main effector caspase substrate – the caspase-3-like activation [60]. Interestingly, we observed that, despite not statistically significant, GK rat brains had a 1.8-fold increment in caspase-3-like activation than age-matched Wistar rats (Fig. 3.10.F). Chronic peripheral administration of Ex-4 was able to reduce caspase-3-like substrate cleavage by 37.2% in GK rats *vs.* placebo-treated GK rats (Fig. 3.10.F). Therefore, these results suggest that Ex-4 may exert its anti-apoptotic effect in T2D rat brain by blunting some intermediary molecule(s) from the apoptotic cascade and it is possible that the increase in autophagy may play a role herein, as discussed later. Amongst such possibly affected intermediary molecules, we analyzed the Bcl2 protein expression and translocation into the cytosol (in basal conditions, the main anti-apoptotic protein Blc2 remains attached to mitochondria, being translocated to the cytosol in the presence of apoptotic signals [79]. Therefore, we measured Bcl2 protein expression in both mitochondrial and cytosolic fractions from brain cortical homogenates. As depicted in the typical immunoblotting image (Fig. 3.10.G), Bcl2 protein was only detected in mitochondrial fractions of all experimental conditions, suggesting that the eventual deleterious conditions associated to T2D were not sufficient to induce its translocation into the brain cortical cytosol in placebo-treated GK rats. Nevertheless, these rats showed a 1.5- fold decrement in mitochondrial expression of Bcl2 compared to placebo-treated Wistar rats (Fig.3.10.G). Conversely, Ex-4 treatment significantly restored Bcl2 protein expression in GK rat brain cortices to values similar to Wistar placebo rats (Fig. 3.10.G). One of the main pro-apoptotic proteins is Bax, that usually relies in the cytosol, being translocated to the mitochondria during the apoptosis process [79]. Although we did not detect any Bax protein expression in mitochondrial fractions from brain cortical homogenates under our experimental conditions (data not shown), its cytosolic expression was similar between Wistar and GK placebo rats (Fig. 3.10.H). Despite no significant changes, Ex-4-exposed GK rats had a 26.4% increment in cytosolic Bax protein expression compared to the GK placebo group. Finally, as cytochrome c translocation from mitochondria (whereby it relies under physiological conditions) to cytosol can be used as an additional apoptosis marker [80], we also evaluated its protein expression levels in both mitochondrial and cytosolic fractions of brain cortical homogenates (Fig. 3.10.I,J). Despite no significant changes between the experimental conditions, neither regarding cytosolic cytochrome c expression (Fig. 3.10.I) nor in mitochondrial expression (Fig. 3.10.J), there was a slightly increased expression of cytochrome c in the cytosol from GK placebo rats (by 9.1% compared to Wistar rats) that was decreased by 15.2 % upon Ex-4 treatment (Fig. 3.10.I).

Overall, these results suggest that the Ex-4-mediated protection against caspase-3-induced apoptosis in T2D GK rat brain cortices may be due (at least partially) to the tendency to retain the anti-apoptotic Bcl2 and the proapoptotic cytochrome c within mitochondria, whereas the proapoptotic Bax remained in the cytosol

A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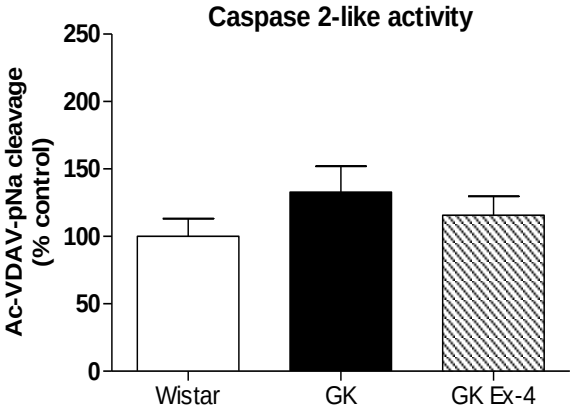
200
150
100
50
0
Wistar
GK
GK Ex-4

B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Ac-VDAV-pNa cleavage (% control)
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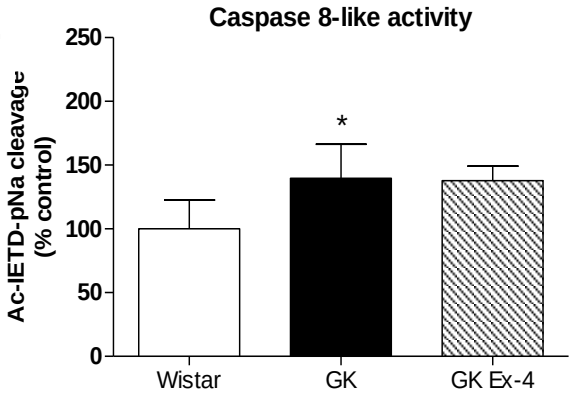
200
150
100
50
0
Wistar
GK
GK Ex-4

C


Caspase 8-like activity
Ac-IETD-pNa cleavage (% control)
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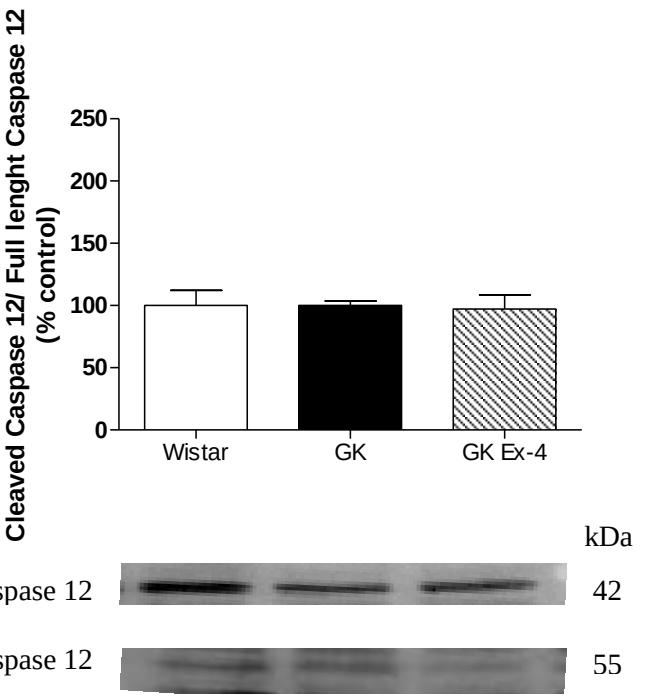
200
150
100
50
0
*
Wistar
GK
GK Ex-4

D


Caspase 12/ Full lenght Caspase 12 (% control)
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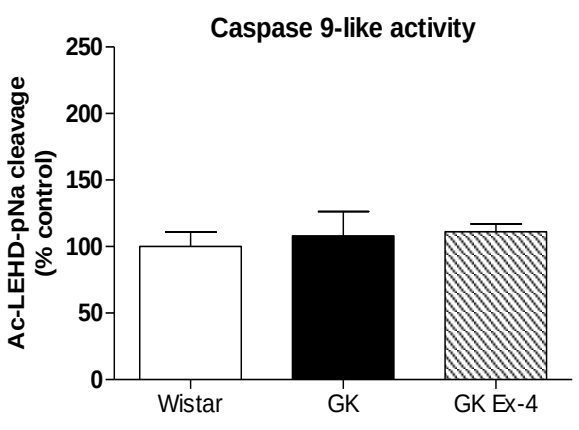
200
150
100
50
0
Wistar
GK
GK Ex-4
Cleaved
kDa
Cleaved caspase 12
42
Full length caspase 12
55

E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Ac-LEHD-pNa cleavage (% control)
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100
50
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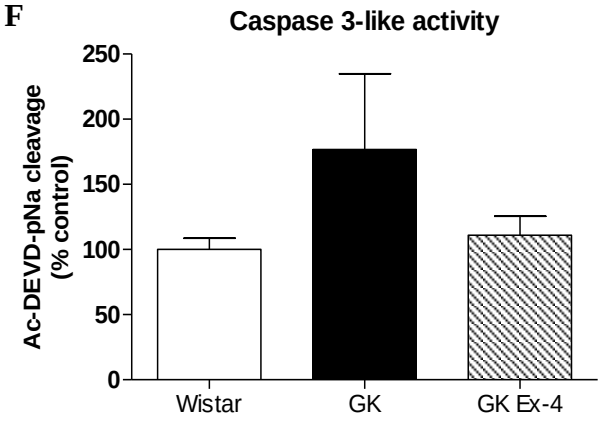
Wistar
GK
GK Ex-4

F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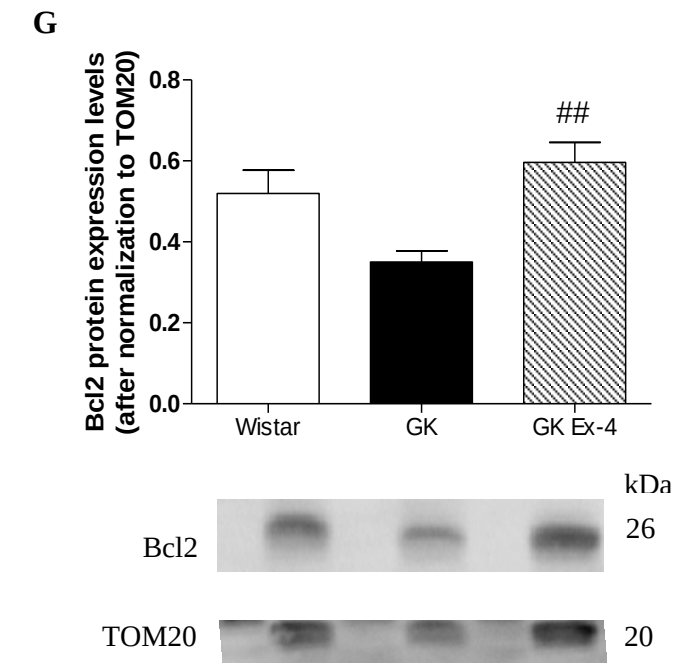
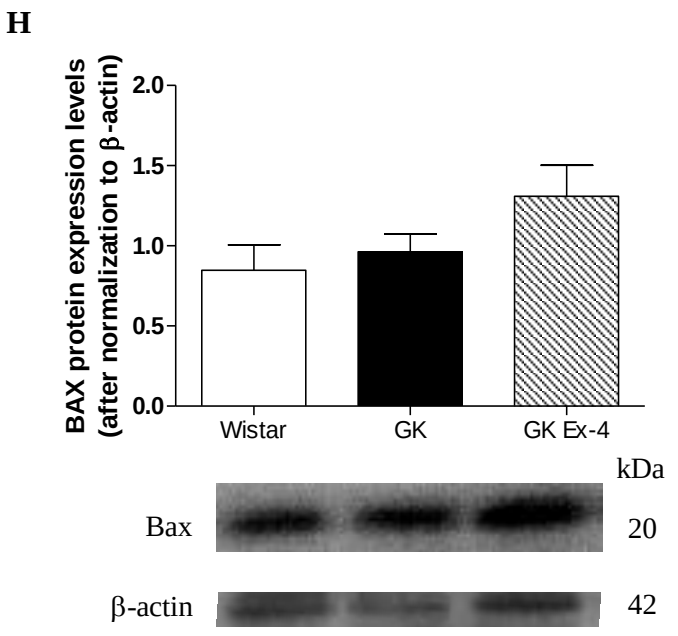
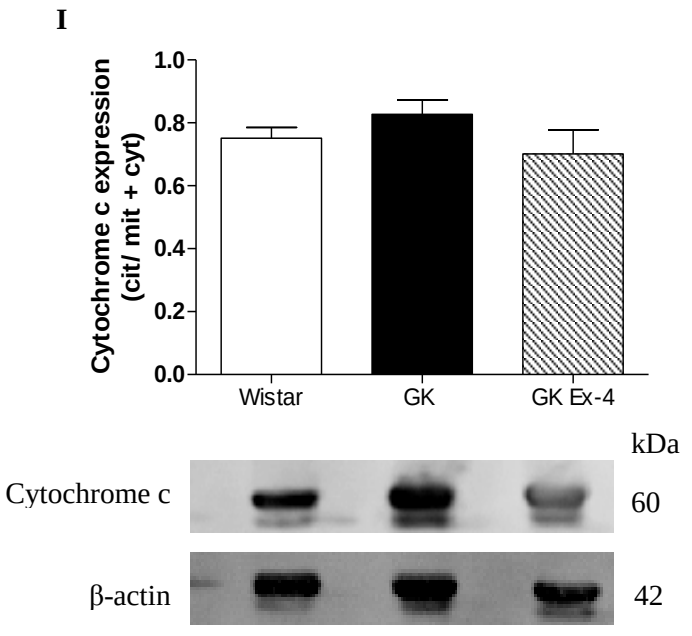
Wistar
GK
GK Ex-4







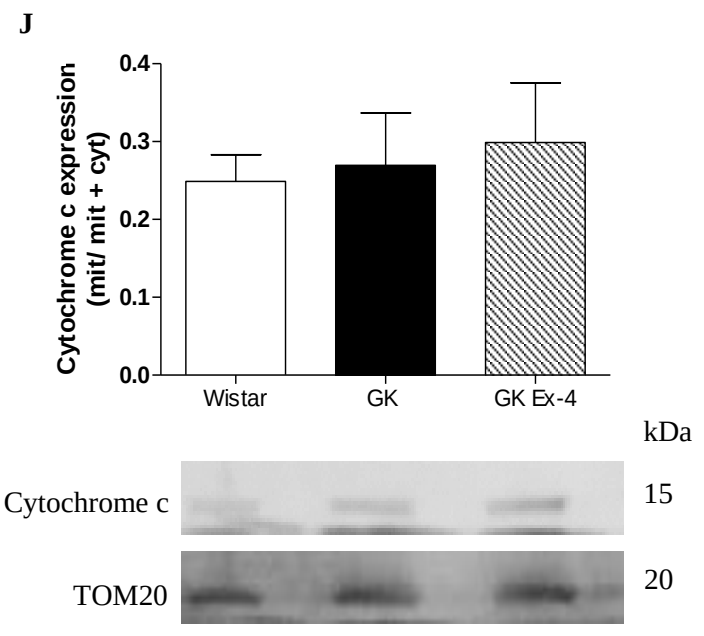


Figure 3.10. Effect of chronic peripheral administration of Ex-4 on T2D rat brain cortical apoptotic markers. After animals' sacrifice, brain cortical homogenates were prepared as described in *Materials and Methods*. Caspases-1- (A), -2- (B), -8- (C), -9- (E) and -3-like activities were measured colorimetrically, at 405 nm, upon the cleavage of their specific substrates (Ac-YVAD-pNA, Ac-VDAV-pNA, Ac-IETD-pNA, Ac-LEHD-pNA and Ac-DEVD-pNA, respectively). Cleaved and full-length caspase-12 (D) were detected by SDS/PAGE (10%) electrophoresis followed by transfer into PVDF membranes and incubation with rabbit polyclonal anti-caspase 12 antibody. Bcl2 (G), Bax (H) and cytochrome c (I,J) translocation was evaluated by SDS/PAGE (10%) electrophoresis of both brain cortical cytosolic homogenates and isolated mitochondrial fractions, followed by transfer into PVDF membranes and incubation with rabbit polyclonal anti-Bcl2 or anti-Bax antibodies. Then, membranes were reprobed with mouse monoclonal anti-β-actin or TOM20. Data from caspases-like activation are expressed as percentage of control and represent the mean ± SEM of six to 10 animals per group. Statistical significance: $^{*}P<0.05$ *vs.* placebo-treated Wistar rats; $^{\#\#}P<0.01$ *vs.* placebo-treated GK rats.

3.9. The role of peripheral administration of Ex-4 on necrotic cell death in GK rat brain cortex

Herewith, we assessed the effect of T2D on rat brain cortical necrotic death and the role for chronic peripheral administration of Ex-4, through the analysis of RIP1 and RIP3, two key regulator proteins of necrosis [104], protein expression levels (Fig. 3.11.A and B). Although there were no significant differences between our experimental groups regarding both RIP1 (Fig. 3.11.A) and RIP3 (Fig. 3.11.B) protein expression levels, a slight, 12% increase in the expression of RIP1 protein occurred in Ex-4-treated GK rat brain cortex (Fig. 3.11.A). These results suggest that necrotic cell death may not underlie T2D-associated brain cortical damage.

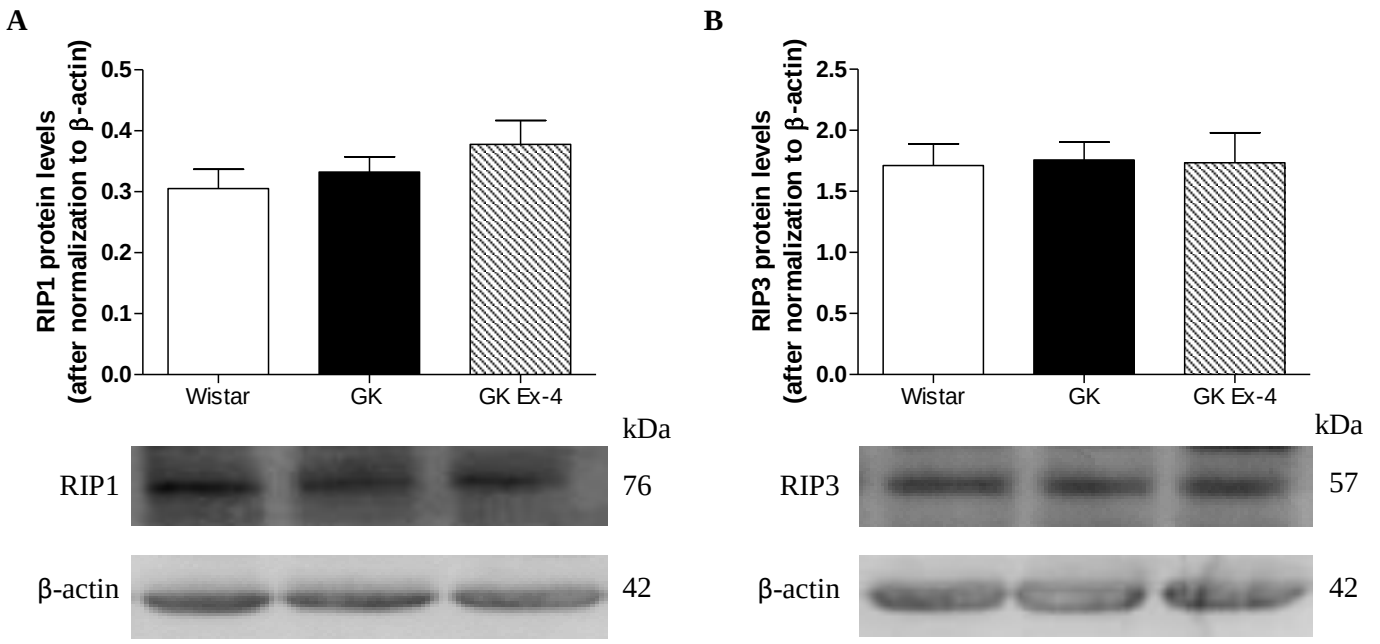


Figure 3.11. Effect of chronic peripheral administration of Ex-4 on T2D rat brain cortical necrotic markers. After animals' sacrifice, brain cortical homogenates were prepared as described in *Materials and Methods*. RIP1 (A) and RIP3 (B) protein expression was evaluated by SDS/PAGE (10%) electrophoresis of brain cortical homogenates, followed by transfer into PVDF membranes and incubation with rabbit polyclonal anti-RIP1 and rabbit polyclonal anti-RIP3 antibodies. Then, membranes were reprobed with mouse monoclonal anti-β-actin. Data are expressed as mean ± SEM of six animals per group.

3.10. Effect of peripheral administration of Ex-4 on synaptic function in GK rat brain cortex

As T2D has been described to negatively affect brain synaptic function [124,210], finally we assessed the effect of Ex-4 on brain synapses through the analysis of protein expression of both the post-synaptic domain-95 (PSD-95, a synapse-associated protein located in the post-synaptic membrane [211](Fig. 3.12.A) and the synaptophysin proteins (a major protein of synaptic vesicles [212](Fig. 3.12.B). As depicted in Fig. 3.12.A, although non-statistically significant, the placebo-treated GK rats had 1.3-fold higher PSD-95 protein expression than age-matched placebo-treated Wistar rats, that was further increased by Ex-4 treatment (to 0.13±0.004). Regarding synaptophysin expression (Fig. 3.12.B), we observed that Ex-4 was able to restore its protein expression in T2D rat brains (from 1.74±0.39 in placebo Wistar rat brain to 1.23±0.35 in placebo GK rats and to 1.9±0.48 in Ex-4-treated GK rats). However, none of these differences reached statistical significance. These results suggested that Ex-4 was trying to restore brain cortical synaptic function in T2D GK rats.

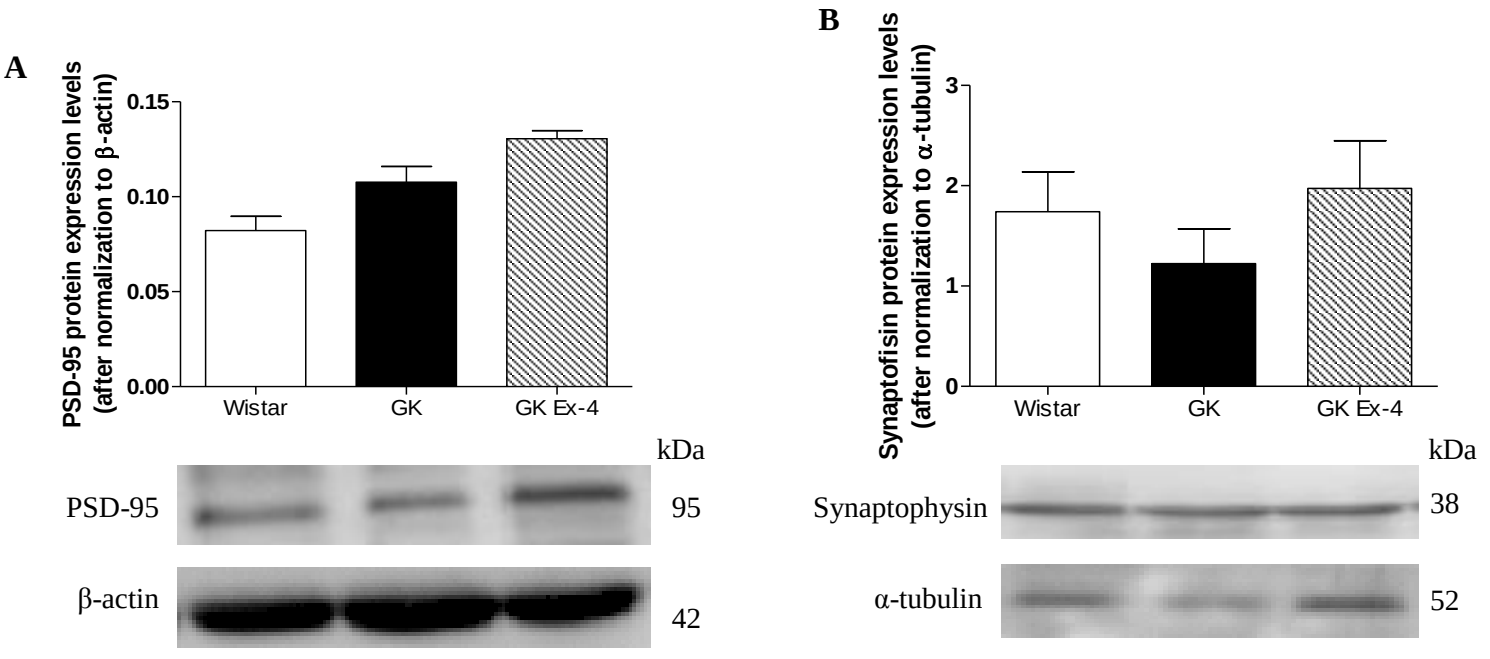


Figure 3.12. Effect of chronic peripheral administration of Ex-4 on T2D rat brain cortical synaptic function. After animals' sacrifice, brain cortical homogenates were prepared as described in *Materials and Methods*. PSD-95 (A) and synaptophysin (B) protein expression was evaluated by SDS/PAGE (10%) electrophoresis of brain cortical homogenates, followed by transfer into PVDF membranes and incubation with rabbit monoclonal anti-PSD-95 and mouse monoclonal anti-synaptophysin antibodies. Then, membranes were reprobed with mouse monoclonal anti-β-actin or rabbit monoclonal anti-α-tubulin. Data are expressed as mean ± SEM of six animals per group.

Overall, our results suggest that Ex-4 is an effective anti-T2D drug able to simultaneously lower GK rats' glycemia and HbA_{1c}, as well as insulin resistance (the main hallmarks of T2D). We also showed that chronic peripheral administration of Ex-4 was able to increase brain cortical GLP-1 levels upon T2D (although this insulinotropic drug was unable to increase plasma and cortical insulin levels). Additionally, despite no effect in brain necrotic cell death, Ex-4 effectively inhibited caspase-3-mediated apoptosis and activated autophagic mechanisms. Additionally, Ex-4 increased synaptic function and decreased brain cortical oxidative stress upon T2D. Although the differences between experimental groups of animals sometimes did not reach statistical significance, the tendencies observed herein appear to be promising and therefore deserve further clarification, *e.g.*, by improving the number of samples analyzed.

CHAPTER 4

DISCUSSION

Discussion

T2D is a growing health care issue and a modern epidemic, thus rendering the discovery and/or the implementation of therapeutic strategies for its prevention and/or management of the outmost socioeconomic relevance. Given that hyperglycemia is a hallmark of T2D [137], a good glycemic control appears to be crucial to reduce the risk for the development of diabetic complications (particularly those affecting the CNS, as dementia and AD). As such, the development of new glucose-lowering agents for the treatment of T2D has been an imperative issue in the recent years. In this regard, as GLP-1 is the physiological responsible for most of the incretin effect [184] in non-diabetic individuals, but its action is highly limited by its vulnerability to degradation by DPP-IV (whose activity is coincidentally increased in T2D), a huge effort has been recently made to develop incretin mimetics (more resistant do DPP-IV degradation) or incretin receptor enhancers (that overcome the deficit in incretin system) to treat T2D. Amongst these drugs, we can consider both GLP-1 mimetics/GLP-1R agonists (as Ex-4) and DPP-IV inhibitors [146]. Ex-4 is one of the most widely clinically used and best studied incretin-based anti-T2D therapy, that acts as an agonist of the GLP-1R [180]. This peptide is obtained from lizard's saliva and is resistant to DPP-IV [176]. Importantly, due to its high lipophylicity, peripherally-administered Ex-4 rapidly diffuses through the BBB into the CNS [183], whereby it has been increasingly shown to exert some neuroprotective effects [213] and, inclusively, led to the development of clinical trials aiming at its use against AD (ClinicalTrials.gov Identifier: NCT01255163). With all this in mind, we hypothesized herein that Ex-4 restores the T2D-associated alterations in intracellular quality control mechanisms, neurodegeneration and death, thereby improving synaptic function and, ultimately, cognitive function. Therefore, we aimed to study the effect of chronic, continuous peripheral administration of Ex-4 in T2D-related pathological features and other associated risk factors, as well as in middle-aged rat brain cortical oxidative stress and synaptic (dys)function markers, cellular quality control (autophagic) mechanisms and, finally, in neurodegeneration and cell death mechanisms.

Regarding the impact of chronic peripheral treatment with Ex-4 on T2D pathology hallmarks, our observation that Ex-4 did not rescue the decrement in body weight in middle-aged GK rats suggested that, contrary to other "traditional" anti-T2D drugs [139], Ex-4 did not induce weight gain, neither had it the anorectic effect described by others. The observation that T2D GK rats had lower body weight than the age-matched Wistar controls was in line with our previous reports on an already decreased food intake and body weight [210,214], as well as with the description of GK rats as a non-obese T2D model [215]. Regarding Ex-4, Young *et al.* (1999)[216] reported that a chronic 5-6 week exposure to Ex-4 (either one-single dose and/or multiple dose treatment) decreased both food intake and body weight in the obese Zucker diabetic fatty (ZDF) rats (hyperglycemic, insulin-resistant, obese, dyslipidemic rat models that succumb to β-cell failure at 12 to 14 weeks of age) [181]. Similar results were described in both obese and lean Zucker rats, as well as in normal rodents consuming a high fat diet, when submitted to a chronic intracerebroventricular Ex-4 treatment [217,218]. Moreover, Ex-4 also successfully decreased body weight in a randomized clinical trial comparing once weekly *vs*. twice daily treatment [219].

Importantly, our observations that Ex-4 treatment lowered occasional glycemia, HbA_{1C} and HOMA-IR, and restored the rate of blood glucose clearance in middle-aged T2D rats, strongly suggested that Ex-4 was able to counteract the main T2D peripheral hallmarks in these animals: the hyperglycemic/glucose-intolerant and the insulin resistant profiles. These results were in accordance with our previous reports on the increased glycemia

and HbA_{1C} (despite the lower food intake) in the 6 to 7 month-old GK rat model [124,214]. Additionally, our results match those describing an Ex-4-mediated decrement in blood glucose levels and HbA_{1c} [175]. Indeed, Tourrel *et al.* (2002) [220] observed that 2 month old T2D GK female rats that were given Ex-4 during their pre-diabetic period (post-natal days 2-6) had a notably decreased plasma glucose level than their untreated counterparts [220]. Moreover, Young *e al.* (1999) [216] described that an *in vivo* intraperitoneal administration of a single dose of Ex-4 to hyperglycemic *db/db* mice (a model of T2D) potently reduced reducing plasma glucose, whereas chronic intraperitoneal administration of this drug (for 5 weeks, twice daily or for 6 weeks, once or twice daily) to male ZDF rats resulted in a significant decline in HbA_{1C}. Similar effects of Ex-4 were provided by Szayna *et al.* (2000)[221] in HbA_{1C} levels from Zucker fatty rats submitted to a 42 day treatment. Interestingly, in a 30 week-randomized clinical trial, Drucker *et al.* (2008) [219] reported that in patients treated with exenatide once a week changes in HbA_{1C} were considerably higher than in those given exenatide twice a day. Interestingly, given that GK rats have been previously described to be mildly hyperinsulinemic and insulin resistant [202], it is not surprising that we observed an increase in plasma insulin levels in untreated rats. Moreover, as Ex-4 is an insulinotropic drug, *ie*, one of its primary effects is to stimulate insulin secretion [203], it is plausible that, despite no significant improvements in plasma insulin levels after treatment (probably due to the already increased insulin in this strain even before drug exposure), an Ex-4-mediated increase in insulin sensitivity might have occurred, as reported in other rodent models of T2D [216,222].

Regarding the impact of Ex-4 on blood cholesterol and triglycerides levels, some controversy exists, as some authors described the drug was able to reduce only the cholesterol levels [181], whereas in others it diminished both cholesterol and triglycerides [219]. In the present study, we observed that Ex-4 did not change blood cholesterol levels and, although not significantly, decreased blood triglyceride levels in GK rats. Similarly, the reports on the effect of Ex-4 in blood pressure are also controversial, ranging from an increased blood pressure after a chronic treatment [223] to a reduction after a once weekly administration [161].

Although no significant changes between cohorts were observed in terms of brain weight, thereby suggesting that middle-aged GK rats did not suffer from macroscopically-detectable brain atrophy and that Ex-4 treatment had no effect herein, previous studies reported that T2D leads to a pronounced brain atrophy and, therefore, to a lower brain weight [2].

As previously described, Ex-4 is a GLP-1R agonist able to cross the BBB and reach the brain intact and, also relevant herein, its receptors are highly expressed in brain [224,225]. In line with this, we first hypothesized that either the peripheral Ex-4 administration in T2D rats would *1)* decrease the secretion of the endogenous GLP-1 due to a negative feedback mechanism, thus decreasing its brain cortical levels, or *2)* it would further stimulate GLP-1 secretion and increase its levels in brain. As from the Fig. 3.5.A, we can infer that the chronic subcutaneous delivery of Ex-4 for 28 days was sufficient to overcome the tendentially lower brain cortical GLP-1 levels in T2D rats, thus suggesting that this anti-T2D drug may stimulate the synthesis of endogenous GLP-1 (either peripherally and/or centrally). Surprisingly, as this was not accompanied by significant changes in GLP-1R protein expression levels between cohorts, it is plausible that, without treatment, T2D rat brain might be suffering from depletion in GLP-1 levels rather than an inhibition of its receptors. However, this needs to be further clarified, *e.g.*, by analyzing if GLP-1R activities are significantly different between groups.

Given the above-mentioned insulinotropic effect of Ex-4 and the increasingly suggested role for insulin resistance as a possible common molecular link between T2D and AD [15,112], we determined brain cortical insulin levels. To our surprise, despite the previously mentioned increase in plasma insulin content in T2D

compared to the age-matched non-T2D rats, its brain levels were significantly lower in the GK rat cohort and, similarly to the periphery, Ex-4 was not able to rescue brain cortical insulin content in GK rats. This suggested that, despite its well known insulinotropic effect (that was not observed peripherally under our experimental conditions); Ex-4 also did not stimulate insulin transport from the periphery into the GK rat brain nor its local brain synthesis. As with plasma, an alternative hypothesis was that Ex-4 could be increasing brain cortical insulin sensitivity instead. However, as no significant changes were observed in the activation profile of brain cortical IR (as given by the phospho-tyrosine/IR), it seems unlikely that Ex-4 was improving insulin sensitivity in GK rat brain. Rather, our results appear to point towards an insulin/IR-independent effect of Ex-4 in T2D rat brain (at least in cortical region). In line with this, we also observed that Ex-4 was able to restore GK rat brain cortical glucose levels to nearly those of age-matched Wistar placebo ones, thus protecting against the described damaging effects of T2D-associated high glucose levels in brain [226]. To further support this hypothesis, we then analyzed the effect of this drug on brain oxidative markers. Oxidative stress is a highly dynamic process that arises from an imbalance between the increased formation of ROS and/or the decrement in antioxidant defenses, being the brain one of the most exposed and vulnerable tissues to the highly deleterious oxidative damage [205]. Such CNS vulnerability is due to its high content on the easily-oxidizable polyunsaturated fatty acids, in transition metals (as iron and copper) and ascorbate, its high metabolic rate and to its lower content in antioxidant defenses [205]. Interestingly, although no statistical differences were observed between the experimental groups, the Ex-4-treated diabetic rats had lower cortical levels of both lipid and DNA oxidation markers (TBARS and 8-OHdG, respectively). These results are in agreement with our previous studies, whereby no significant differences in lipid oxidation were reported between middle-aged Wistar and GK rat brains under basal conditions, whilst under an additional detrimental condition (*e.g.* the induction of an exogenous oxidative injury) the T2D rat brains were either less vulnerable (at 6 month old) [210,214] or unaffected (at 7 months of age) [124]. Importantly, such decreased brain vulnerability of GK rat brains appeared to be independent from synaptosomal fatty acid composition or the antioxidant enzymes superoxide dismutase, glutathione oxidase and glutathione reductase; rather, it could be due to a higher content on the endogenous scavengers oxidized glutathione and vitamin E [214]. Our present results on the Ex-4-mediated partial decrease in brain lipid and DNA oxidation was in accordance with the observed Ex-4-associated control of DNA oxidative damage and 4-hydroxy-2-hexenal (a major lipid peroxidation product formed by n-3 polyunsaturated fatty acids) levels upon ischemia/reperfusion injury-induced oxidative stress [227].

Autophagy is a catabolic mechanism that, physiologically, contributes for cell survival due to the degradation of old and damaged proteins; nevertheless, some controversy exists on the role of autophagy after brain damage, as it has also been implicated in cell death [29]. From our results on the protein expression levels of the commonly used autophagic markers LC3-II (expressed in autophagosomes [48]), beclin-1 and PI3K class III (both regulatory proteins that form an initiator complex in the autophagic process [28,49]), as well as from the phospho-mTOR/mTOR (a key regulator of autophagy), we could infer that the autophagic process could be (at least partially) more activated in brain cortices from the Ex-4-treated GK rats and, thereby, constituting a protective strategy against T2D-related brain degeneration and death.

Ex-4 has been shown not only to protect pancreatic β-cells against apoptosis, but also to increase their mass [213,220]. Interestingly and partially in line with a possible induction of a "protective" autophagy by Ex-4 are our observations of a tendentially lower activation of the initiator caspases-1 and -2 (belonging to the intrinsic pathway) in GK treated rat brain cortices which, together with their tendency to retain the anti-apoptotic Bcl2

and the proapoptotic cytochrome c within mitochondria (whereas the "inactive" proapoptotic Bax remained in the cytosol), as in physiological conditions [67,80,81], might have contributed for the lower caspase-3 activation (the major effector caspase) reported in Ex-4-treated GK rats. Notably, one possible explanation for the apparent Ex-4's efficacy only in the later (rather also in the initial) stages of the apoptotic cascade relied on a "controlled", physiologically-like activation of autophagy in our rodent models. Indeed, Hou *et al.* (2010) [228] observed a simultaneous *in vitro* co-localization of the LC3-II punctuates with processed fragments resulting from caspase-8 activation in an HCT116 cell line of human colon carcinoma). This suggested that a similar degradation mechanism could be responsible for LC3 and cleaved caspase 8 and, in the presence of lysosomal degradation inhibitors, the expression levels of both cleaved caspase-8 and/or punctuate LC3 were maintained [228]. Therefore, it was possible that, in our conditions, the cleaved caspase-8 was engulfed by the autophagolysosomes upon Ex-4 treatment and, thus, we could not observe any direct effect of the drug on caspase-8 activation. To our knowledge, despite no further information on a similar effect on other caspases, we cannot rule out that Ex-4 activated autophagy may (at least partially) eliminate some of the initial activated caspases, thereby interfering with the progression of the apoptotic cascade in GK rat brains. Importantly, these results appear to be in accordance with the previously described neuroprotection conferred by Ex-4 against apoptosis [206,229,230]. Although the endoplasmic reticulum-related caspase-12 has been increasingly suggested to play a role in AD-associated apoptosis [97], it seems unlikely to play a role in our conditions. Additionally, our results on necrotic cell death further suggest that necrosis may not underlie T2D-associated brain cortical damage, as given by the protein expression levels of RIP1 and RIP3 (two key regulator proteins of necrosis [104]).

T2D has been also described to negatively affect brain synaptic function [124,210]. Accordingly, our results suggest that Ex-4 was able to (partially) restore brain cortical synaptic function in T2D GK rats. These results were in accordance with the recent observation that Ex-4 promoted LTP, formation of memory and learning skills, as well as to restore and enhance cognition in AD patients [231].

CHAPTER 5

CONCLUSION

Conclusion

In the present study, we hypothesized that Ex-4 restores the T2D-associated alterations in intracellular quality control mechanisms, neurodegeneration and death, thus improving synaptic function and, ultimately, cognitive function. From the previously described results, we can conclude that chronic peripheral administration of Ex-4 ameliorated the main pathological features of T2D: hyperglycemia, glucose-intolerant and insulin resistant profiles in middle-aged T2D male GK rats.

Regarding the effect of peripheral Ex-4 administration in GK rat brain cortex, it is possible that the restoration of GLP-1 levels might be accompanied by an improvement in GLP-1R activity in brain. However, this deserves further clarification. Conversely, as Ex-4 did not interfere with brain cortical insulin levels or its receptor activation, it is likely that the subsequent effects of Ex-4 in GK rat brain might not involve insulin/IR. Such Ex-4 effects included a restoration of brain glucose levels, a tendency to protect against lipid and DNA oxidation and to activate the autophagic process, probably as a protective strategy against the subsequently reported T2D-related caspase-3-mediated death in placebo-treated GK rats. Hopefully, this would culminate in a slightly improved brain cortical synaptic function in Ex-4-exposed T2D rats.

Overall, from our results we can conclude that chronic, continuous, peripheral Ex-4 exposure may have a beneficial neuroprotective impact in middle-aged, non-obese T2D GK rat brain cortices, thus constituting a protective strategy against T2D-related brain degeneration and death.

CHAPTER 6

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